2013-01-09

Centritubing: Using Centrifugal Force to Create Self-Assembled Tubular Tissue Constructs

Craig Jones
Worcester Polytechnic Institute

Centritubing: Using Centrifugal Force to Create Self-Assembled Tubular Tissue Constructs

A thesis submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Master of Science

December 2012
Submitted by

Craig Michael Jones
Department of Biomedical Engineering

Approved by:

Kristen L. Billiar, Ph.D
Associate Professor
Department of Biomedical Engineering
Committee Member

Raymond L. Page, Ph.D
Assistant Professor
Department of Biomedical Engineering
Committee Member

Marsha W. Rolle, Ph.D
Assistant Professor
Department of Biomedical Engineering
Thesis Advisor

Acknowledgements

I would first like to thank my advisor, Dr. Marsha Rolle for her inspiration and excitement about this project when we first proposed it during our Major Qualifying Project so long ago. I would also like to thank her for her incredible guidance and support throughout the ups and downs of this project.

I would also like to thank my committee members, Dr. Kristen Billiar and Dr. Raymond Page for their valuable feedback and guidance throughout my time at WPI.

Finally, thank you to Zoe Reidinger, Tracy Hookway, Xuyu Qian, John Burford, Laurie Soderbom, Paul Sullivan, Sharon Shaw, Lisa Wall, Jack Ferraro, and Neil Whitehouse whose input and assistance was vital to the completion of this project.

Abstract

With 500,000 coronary artery bypass procedures performed each year in the United States, and only one-third of patients possessing suitable autologous grafts, there is a clinical need for tissue engineered blood vessels (TEBVs). The overall goal of this project was to develop a one-step approach to rapidly produce entirely cell-derived tubular tissue constructs without scaffold materials. To achieve this goal, we developed "centritubing"— a system based on applying centrifugal force to suspended cells to create a tube-shaped cellular aggregate. Briefly, rat aortic smooth muscle cells were injected into cylindrical polycarbonate spinning chambers and then spun to apply centrifugal force, which pelletted the cells on the inner wall of the chamber. After three days in culture with growth medium, the cells remodeled to form tissue tubes. In previous work we have shown, in principle, that centritubing produces tubular constructs, however tissue tube production was not consistently achieved. The first objective of this study was to develop modifications to the centritubing device that would lead to consistent lumen diameter, rapid cellular aggregation into a tube construct, and an improved success rate of tube formation. The second objective was to investigate cellular parameters that contribute to tubular tissue construct formation using centritubing.

Prior to changes in manufacturing of the centritubing device and culture system, the success rate of centritubing was inconsistent. After these changes, the success rate of tubular construct formation improved to 85% (11/13). Noteworthy modifications to the centritubing device included the addition of a central mandrel as a substrate for tissue contraction, development of a smoother seeding surface, and manufacture of a reusable culture chamber. The results of this study support the proof of concept for centritubing as a device for rapid production of tubular tissue constructs and provide insight for future progress using the centritubing methodology.

Contents

Table of Figures

Table of Tables

Chapter 1 – Introduction

In recent years, it has become apparent that tissue engineering can provide clinically viable solutions to regenerative medicine (Dinh, 2006; Ehrenreich, 2006; McAllister, 2009; Hibino, 2010). While these complex living models mimic natural tissues, there remains an unmet need for rapid assembly of cells to form these tissues.

Due to its clinical significance, the small diameter blood vessel has garnered significant attention in tissue engineering. Numerous strategies have been employed to create tissue engineered blood vessels (TEBVs) using synthetic scaffolds (Niklason, 1999; Xue, 2003; Nasseri, 2003; Hibino, 2005; Soletti, 2006; Lee, 2008; Gong, 2008; Soletti, 2010), natural materials (Hirai, 1996; Swartz, 2005; Isenberg, 2006; Pankajakshan, 2007; Ahmann, 2010; Schutte, 2010), or "scaffold-less" entirely cell-derived extracellular matrix (ECM) (L'Heureux, 1998, Norotte, 2009; Gwyther, 2011). Cell-derived tissues may provide advantages over other methods, including improved mechanical properties (Ahlfors, 2007; Adebayo, 2012) and minimal foreign body response (Laschke, 2009; Ghanaati, 2011). However, many cell-derived matrix (CDM) TEBVs require long culture times, manipulation of tissue, and many cells (L'Heureux, 1998, Norotte, 2009; Gwyther, 2011, CTO). Currently, all TEBVs require a transition period of some sort—whether it is scaffold degradation or tissue fusion—to reach the final state of a cellular-derived tube. Therefore, there is a need for a one-step method of rapid self-assembly to form a scaffold-less cell-derived TEBV.

Our lab has designed and implemented a novel method for creating tubular tissue constructs entirely from self-assembled cells that requires no synthetic scaffold materials (Burford, 2010). These tubular tissue constructs are created by a method we call centritubing, which utilizes centrifugal force to pellet cells onto the inner wall of a cylindrical spinning chamber, allowing them to attach and grow to form a cohesive tissue. A schematic of the centritubing device is displayed in Figure 1.

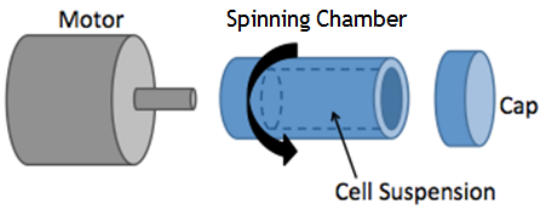

Figure 1 - A schematic representation of the centritubing device. A spinning chamber containing a cell suspension is attached to a motor, which will spin the chamber around its cylindrical axis.

The spinning motion of the motor acts similar to that of a common laboratory centrifuge used for phase separation of cells and media. As shown in Figure 2, a cell suspension inserted within this spinning chamber will be forced away from the central axis, toward the inner wall of the cylinder. This force, along with cellular attachment proteins, will adhere the cells to the spinning chamber surface.

After 3 days in culture the cells formed a cohesive tissue, which contracted off the spinning chamber surface and retained its tubular structure.

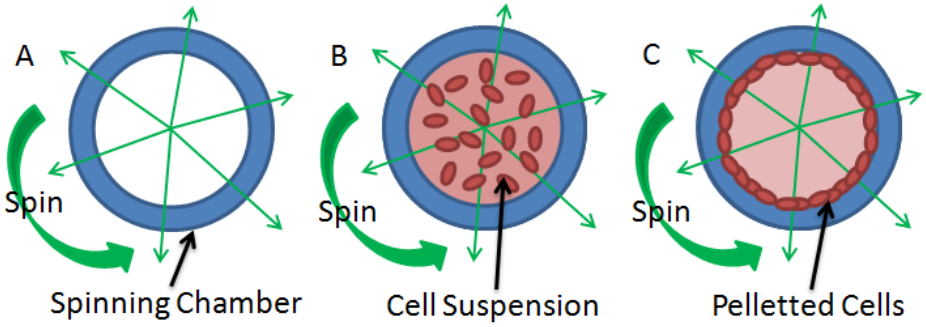

Figure 2 - A schematic representation of centritubing cells. A) The outward centrifugal force applied during centritubing, B) a cell suspension within the spinning chamber, and C) cells pelletted to the spinning chamber wall after centritubing.

We previously demonstrated, in principle, that centritubing can produce cohesive tissue tubes. However, results were inconsistent in terms of tube dimensions and the success of tube formation. The primary goal of the present study was to optimize the existing centritubing technology to rapidly aggregate cells into an entirely cell-derived tubular construct. A secondary goal was to consistently create these constructs with a set lumen diameter.

To achieve these goals, we focused on the following Specific Aims:

Specific Aim 1: **Modify the centritubing device to consistently and rapidly create a cohesive cellular construct with a <6 mm lumen.** To do this, we first identified device design and preparation considerations that lead to tube failure in centritubing. Second, we incorporated design changes, including a central mandrel as a substrate for tubular contraction, a sterile and multi-use culture apparatus, and a smoother seeding surface. Finally, we evaluated these modifications for improved success in tube formation using centritubing.

Specific Aim 2: **Systematically evaluate the cellular processes that could lead to tube formation failure in the centritubing method.** Cellular parameters to be evaluated included adequate cell coverage, cell viability after centritubing, the impact of centrifugal force on cell attachment, and the effects of alternate dissociation methods on cell-cell attachment.

In this project, several centritubing studies were performed, which identified complications that could lead to tube failure. It was found that tube failure was potentially caused by culture conditions, imperfect manufacturing of motor-spinning chamber interface or spinning chamber seeding surface, and the disruption of seeded cells. These studies also introduced solutions, such as a central mandrel, alternate manufacturing methods, and changes in device preparation to improve the rate of success in tissue tube formation. Further studies

provided insight that could identify ideal cellular parameters for future use in centritubing. Overall, the present study provided evidence that centritubing is potentially a viable method for one-step aggregated cellular tube construct creation.

Chapter 2 – Background

This section discusses the background necessary to understand the motivation for this project and the rationale behind the studies that were performed to improve and optimize TEBV formation using centritubing.

2.1 – Clinical Need

In 2011, it was reported that over 16 million Americans had coronary heart disease, with almost 8 million relating to myocardial infarction (heart attack) (Roger, 2011). These problems are most often related to thrombosis or atherosclerosis of the coronary arteries, impairing blood flow and nutrient delivery to the heart muscle. This results in death of the contractile myocytes in the heart wall and failure to efficiently pump blood to the rest of the body. While stents have been implanted in the coronary artery to compact atherosclerotic plaques and clear the path of blood flow, this is only a temporary solution, with 25% of stented vessels experiencing restenosis after surgery (Dangas, 2002). The most effective solution to coronary heart disease is to bypass the coronary artery using a coronary artery bypass graft procedure (CABG) (Cole, 2002; Rodriguez, 2003; Kohsaka, 2008). The "gold standard" bypass conduit is another autologous vessel, such as the saphenous vein or internal mammary artery. This procedure is performed approximately 500,000 times each year in the United States (Mitchell, 2003). Although CABG is successful in delaying the negative effects of coronary artery blockages, it does not treat the cause of the disease and 7% of patients require reoperation within 10 years and 18% within 20 years (Sabik, 2006). Between previous CABG procedures and poor vascular health, almost one third of patients requiring a bypass graft do not have suitable vessels available for harvest (Tu, 1997; Cohn, 2006). Due to this, there is a need for a more reliable, off-the-shelf vascular graft material that can be used for CABG.

Replacement grafts have been attempted using biomaterials, such as Dacron or ePTFE, but these only remain patent in large diameter applications (>6 mm lumen), with poor long-term patency in small diameter applications (<6 mm lumen), including CABG (Pasic, 1996; Basaran, 2003). The ideal vascular graft substitute for CABG should be a living, native-like small diameter blood vessel. TEBVs can potentially provide this ideal substitute.

2.2 – Scaffold Tissue Engineered Blood Vessels

The original theory behind tissue engineering was to create three-dimensional scaffolds that could be seeded with cells and grown into a tissue construct of the desired shape. A scaffold is a 3-dimensional porous solid biomaterial designed to perform some or all of the following functions: biodegrade at a rate of tissue regeneration, promote cell-biomaterial interactions, cell adhesion, extracellular matrix deposition, cell survival, cell proliferation, cell differentiation, and provoke minimal inflammatory response (Freed, 2007). Scaffold-based tissue engineering has been explored extensively for applications in CABG and other vessel-based procedures, allowing the growth of living small diameter blood vessels. The first attempt to create a TEBV was developed by Weinberg and Bell using cells seeded on a collagen scaffold (Weinberg, 1986). Since then, countless degradable synthetic or

natural scaffolds have been designed and seeded with cells to grow TEBVs. Synthetic scaffolds have used a variety of polymers, including poly(ester urethane), poly lactic acid, polyglycolic acid, or polycaprolactone (Niklason, 1999; Xue, 2003; Nasseri, 2003; Hibino, 2005; Soletti, 2006; Lee, 2008; Gong, 2008; Soletti, 2010), while natural scaffolds have mostly utilized collagen or fibrin matrices (Hirai, 1996; Swartz, 2005; Pankajakshan, 2007; Ahmann, 2010; Schutte, 2010). Several of these TEBV models have led to breakthroughs regarding mechanical conditioning of engineered tissue to improve strength (Niklason, 1999) and rapid production of tubular vessels using cells embedded in matrix gel (Syedain, 2011).

These groups have succeeded in creating living small diameter blood vessels. However, an implanted TEBV must have strength similar to the native vessel it will replace to avoid compliance mismatches (Isenberg, 2006). Scaffold-based TEBVs can mimic the mechanical properties of native vessels, but are restricted to seeding upon materials that are biocompatible and biodegradable. Several of the TEBV methods mentioned above displayed poor mechanical integrity when using clinically relevant cells (Soh, 2005; Gong, 2008)—which would be perilous in the CABG application.

Entirely cell-derived tissue engineering methods have been shown to grow mechanically robust tissues without the drawbacks of a synthetic scaffold (Ahlfors, 2007; Peck, 2012).

2.3 – Entirely Cell-Derived Tissue Engineered Constructs

In 1998, L'Heureux, et. al. introduced their entirely cell-derived tissue engineered blood vessel, which is currently in clinical trials and has become the foundation of Cytograft Tissue Engineering Inc. (L'Heureux, 1998). In this method, sheets of fibroblasts are cultured on standard tissue culture plastic in the presence of ascorbic acid for increased collagen synthesis. Sheets are then rolled around a cylindrical support and allowed to mature into a tube. These vessels have displayed burst pressure strength greater than native saphenous veins—the gold standard for CABG (Konig, 2009).

The prospect of entirely cell-derived tissue engineered constructs has already proven successful in clinical and commercial applications (Dinh, 2006; Ehrenreich, 2006; Lysaght, 2008; McAllister, 2009; Hibino, 2010). For example, Genzyme's EpiCell is a living sheet of epithelial cells for wound healing. Outside the clinic, various methods have been developed to grow entirely cell derived rings and spheres over non-adhesive substrates (Napolitano, 2007; Gwyther, 2011, JOVE). Due to their rapid self-assembly, tissue rings have shown great promise as models for analyzing tissue strength, investigating ECM, and optimizing media formulations (Hu, 2010; Adebayo, 2012). Tissue structures such as these have further been cultured in close contact and displayed remarkable tissue fusion into complex shapes, such as tubes (Rago, 2009; Norotte, 2009; Gwyther, 2011).

While these options are promising, it is still unclear how long tissue microstructures should be cultured before optimal tissue fusion. Extended culture is required to create the initial building block tissues and then also fuse them together. The total process of fusion can take up to 2-3 months for tissue maturation (L'Heureux, 2006). Furthermore, in all cell-derived constructs mentioned, there is a risk regarding delamination of tissue layers and weak points where tissue has fused (McAllister, 2009).

There is still a need for an entirely cell-derived tubular tissue construct that can be rapidly aggregated into a tubular shape for a one-step production process.

2.4 – Our Solution: Centritubing

Our lab has developed an alternative method to create a tubular tissue construct by cellular self-assembly without tissue fusion or a scaffold. The concept is derived from the operating principles of a standard laboratory centrifuge, which spins at high velocity and uses centrifugal force to cause phase separation of a heterogeneous suspension (in this case, a suspension of cells in culture media).

Centrifugation: Translation to a Tubular Application

Centrifugation is typically performed in a conical test tube, in which the cells are "pelletted" to the tip of the tube in an aggregated clump. In this application, the distribution of cells within the pellet is dependent on the direction of the force in the test tube and the shape of the seeding surface. If you imagine a flat surface with a centrifugal force that is perpendicular to the face of the surface, in theory, a pellet the shape of this surface should result (Figure 3).

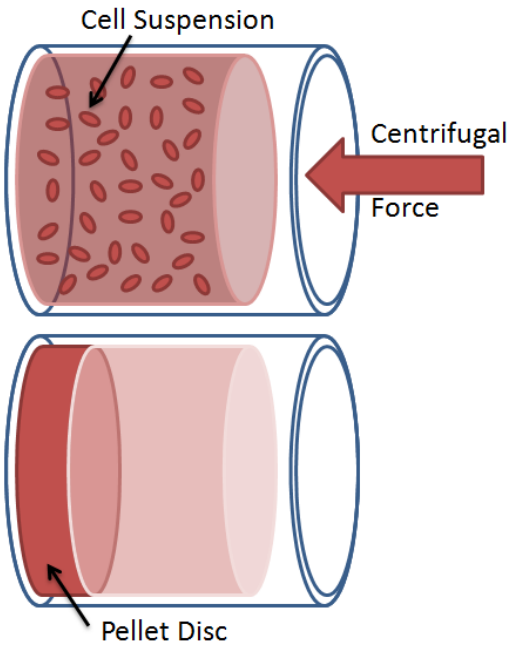

**Figure 3 - Centrifugation of a cell suspension perpendicular to a given surface.
Centrifugal force should result in a pellet of cells upon that surface.**

Taking the theory one step further, translating this force to a cell suspension within a cylindrical (tubular) chamber should result in the same phase separation and force holding cells to the cylinder inner wall (Figure 4).

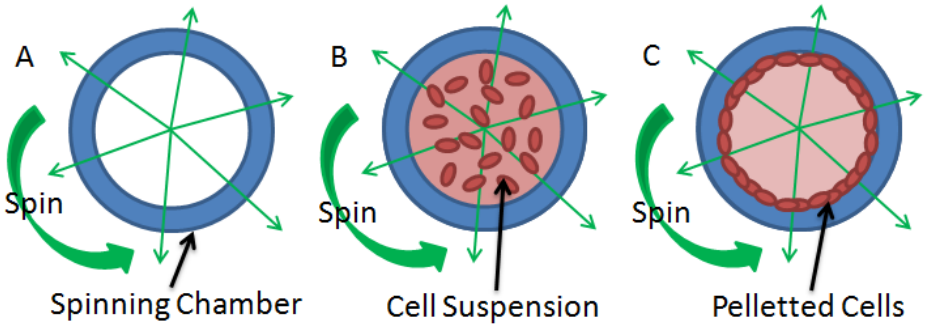

Figure 4 - A schematic representation of centritubing cells. A) Centrifugal force acts outward inside a cylindrical spinning chamber. B & C) A cell suspension within such a spinning chamber should be pelletted to the surface, similar to a centrifuge.

The theory of applying centrifugal force to a cylindrical application is the primary concept behind centritubing—the process of creating a tubular, aggregated cellular construct using centrifugal force. The force applied during standard laboratory centrifugation is greater than the force used in our method (Burford, 2010).

Previous Studies

To create tubular tissue constructs by centritubing, a suspension of cells was spun axially inside a custom manufactured plastic tube (the spinning chamber). The spinning chamber was created by drilling into a polycarbonate rod to create a container for the cell suspension. This container was capped with silicone glue to prevent leakage. In addition, a connection was designed to fit the spinning chamber onto a motor. The set up for this device is shown in Figure 5. A cell suspension of vascular smooth muscle cells (SMCs) was used to replicate the primary functional media layer of a blood vessel. This suspension was injected through the cap and the spinning chamber was spun upon the motor. The resultant centrifugal force pushed the cells out of suspension (Figure 6A) and onto the wall of the tube in a theoretically uniform distribution (Figure 6B). However, in practice, there is potential for a non-uniform cell suspension and a non-uniform seeding surface, which could result in a non-uniform distribution of cells upon the surface.

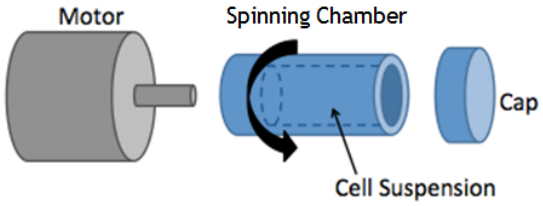

Figure 5 – A Schematic of the centritubing device. The device for centritubing is set up with a capped spinning chamber filled with a homogenous cell suspension. The spinning chamber attaches directly to the motor shaft.

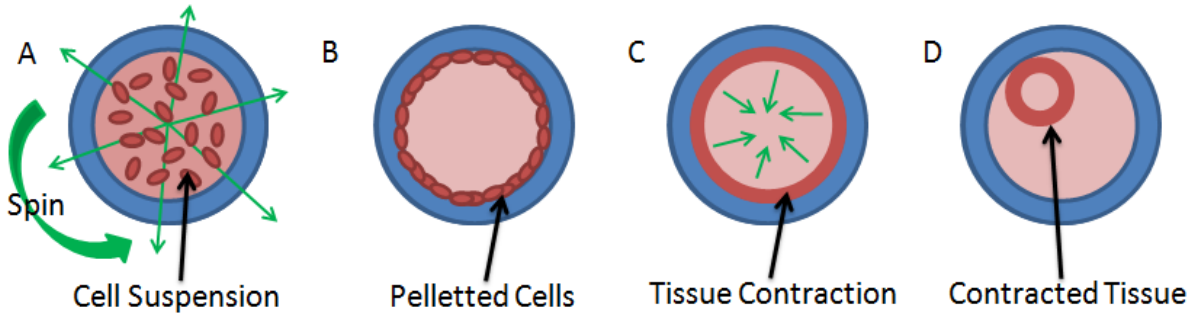

Figure 6 – A schematic of tissue tube formation in centritubing.
A) Centrifugal force will push the cells toward the spinning chamber wall during centritubing. B) Cells are separated from the media and pelletted to the spinning chamber wall. C) Cells attach to one another and form a cohesive tissue, which begins to contract. D) Contracted tissue retains its cohesive tubular shape.

After spinning, the spinning chambers' caps were removed and they were incubated in reduced serum media. After three days, the cells formed a cohesive tissue construct (Figure 6C), which had contracted off the spinning chamber wall (Figure 6D). A bioreactor was designed to accomplish this goal, constructed from common lab supplies: a 50 mL conical tube, a cryovial, a 3 mL syringe, a culture flask cap, and a three-way stopcock. These were connected such that the 50 mL conical tube would rest on its top, inverted, while the spinning chamber is held in the cryovial. This allows the spinning chamber to stand vertically during culture (Figure 7). The three way stopcock and syringe are used to add and remove media, while the culture flask cap replaces the bottom of the conical tube to allow oxygen and CO_2 diffusion between the incubator and the bioreactor.

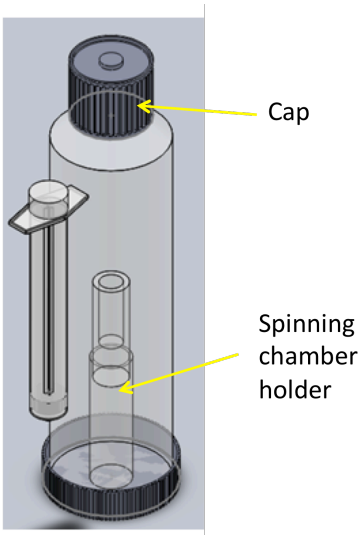

Figure 7 - A 3-D representation of the conical culture apparatus.
This was used for vertical culture of spinning chambers with tubular tissue constructs.

After culturing, the cellular tube (Figure 8) could be removed from the polycarbonate spinning chamber with forceps. As expected, after only three days of culture, collagen was not detected by Masson's Trichrome staining, and Hoescht staining revealed a high density of smooth muscle cells.

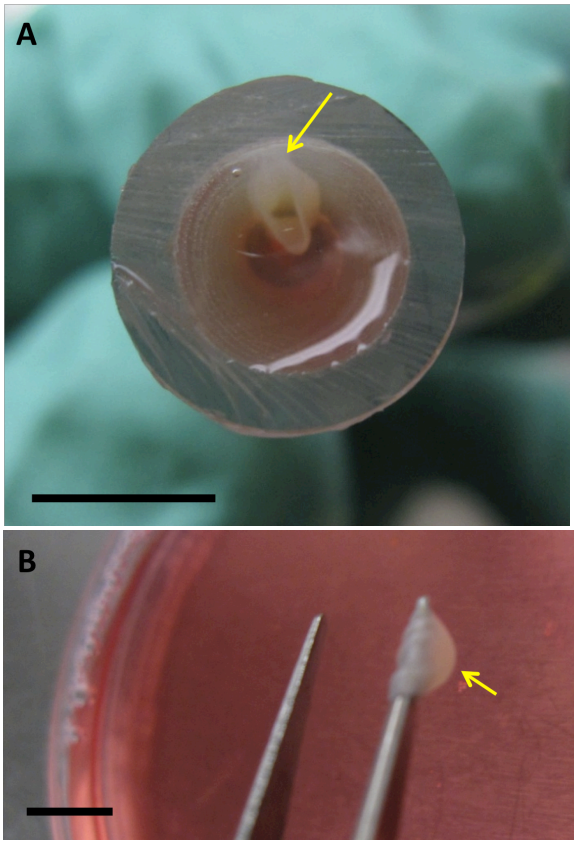

Figure 8 – Three day tissue tubes. A) After three days in culture SMCs formed tissue and contracted into a tubular shape. Scale bar 3 mm. B) Tubular constructs displayed weak structure. Scale bar 1 cm.

Using this one-one step centritubing process, our device quickly assembled cells into tubular tissue constructs, because the cells aggregated in a tubular shape. The materials for the centritubing device are inexpensive and the protocol requires a short production time (~ 4 days) for tube creation (Burford, 2010). Despite the success of this device, there was still little control over the resulting tissue, which was inconsistent in size, shape, success of tissue creation, and tissue was difficult to harvest without damage. In order to utilize the full potential of centritubing, it is important to understand the different forces and cellular parameters that play a role in tissue tube formation and consistency.

2.5 – Forces in Centritubing

In the previous section, centrifugal force was the term used for the movement of cells toward the bottom of the test tube or the spinning chamber wall. The true causes of this centrifugal force and the cells pelletting to the surface of the spinning chamber are their own inertia, the inertia of the fluid

medium, and the radial motion of the surface towards them. Because cells are denser than the medium, they will be forced toward the pelletting surface. Once a cell reaches the wall of the chamber, there are three prominent forces acting on the cell. There is inward centripetal force of the chamber surface, there is the reactive centrifugal force of the media acting on the cell, and there is the shear stress of the spinning media flowing over the stationary cell (Figure 9).

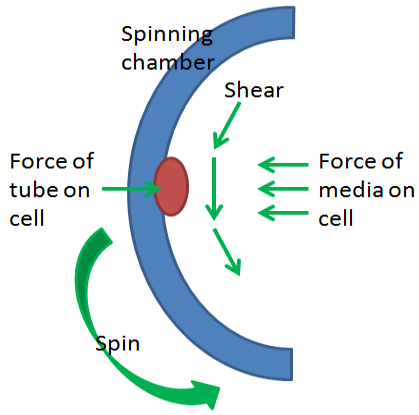

Figure 9 - A schematic of the forces acting on a cell during centritubing. These include the centripetal force of the tube on the cell, the reactive centrifugal force of the media on the cell, and the shear force of the spinning media on the cell.

As shown in the schematic, the centripetal and centrifugal forces act in compression on the cell. While these forces can theoretically be calculated, it is more useful to think of the amount of force used to pellet the cells in terms of acceleration due to gravity in rotational motion.

Calculating Relative Centrifugal Force

The primary unit of force in centritubing and centrifugation is relative centrifugal force (RCF), which is expressed as multiples of acceleration due to gravity (1 xg = 9.81 m/s^2). The formula for relative centrifugal force is shown below (BHG Hermle Z320 Laboratory Centrifuge Manual):

$$g = 11.18 \times r \times \left(\frac{n}{1000}\right)^2$$

g = relative centrifugal force (RCF), i.e relative to the acceleration of the earth which has the constant value g = 9.81 m/s^2

r = spinning radius in cm

n = revolutions per minute (RPM)

For example: using the specifications for centritubing (a radius of 0.3175 cm and the revolutions per minute of 11,000) it was calculated that a force of 429.5 xg is applied to the cells. Using the

specifications for a standard centrifuge with a 16 cm radius operating at 4,000 rpm, a resultant force of 2,860 xg was calculated. This suggests that cells will not be significantly harmed in centritubing because less RCF is applied than when cells are centrifuged during routine cell culture and passaging.

2.6 – Effects of Mechanical Forces on Cells

As described previously, several mechanical forces are applied upon the cells during centritubing. There is the force pushing the cells against the spinning chamber wall and also the shear force of the media during spinning. Similar forces have been shown to initiate changes in the behavior of various cell types, including SMCs.

Centrifugation, specifically, has been used as a compressive force to differentiate cells (Johnstone, 1998). Bone marrow progenitor cells, such as mesenchymal stem cells (MSCs), have been centrifuged at up to 600 xg for 5 minutes and cultured in chemically-defined chondrogenic medium for 7-21 days (Zhang, 2010). The resulting cell masses have shown RNA and histological similarities to natural chondrocytes. The magnitude of compression can also distinguish differentiation down a chondrogenic or osteogenic lineage (Pelaez, 2012). On the other hand, cyclic compression of synovial cells has been shown to increase the production of matrix metalloproteinases (MMPs) that break down collagen (Akamine, 2012).

Cell differentiation is also affected by shear, stain, and other surface or environmental factors. Shear stress by fluid flow has been used to initiate differentiation of MSCs or marrow stromal cells into endothelial cells or SMCs (Yamamoto, 2005; Kim, 2011; Khan, 2011). Constant flow with pulsatile pressure applied, meant to mimic hemodynamics, showed improved SMC markers such as myosin heavy chain (MHC) in stromal cells (Kobayashi, 2004). Similarly, cyclic strain—similar to pumping of blood through vessels—increased SMC markers in MSCs (Shimzu, 2008). Along with flows and strains, other mechanical stimulators, such as substrate stiffness and topography, have impacted stem cell differentiation (Discher, 2005; Joy, 2011; Li, 2011; Tee, 20011).

Several studies have also investigated the influence of mechanical strain and shear directly on SMCs. SMCs are a unique cell type because they have a distinct plasticity in their phenotype—they can switch between synthetic (proliferative) or quiescent (contractile) and there are distinct protein markers for each (Aikawa, 1997). In the body, SMCs experience cyclic strain every time the heart beats, however, they will only experience high shear stress during vascular injury in which they are exposed to blood flow. The effects of shear stress on SMCs have varied and are not well characterized, but it is generally accepted that the glycocalyx surrounding the SMC cell membrane is heavily comprised of heparin sulfates that help modulate the mechanotransduction of shear stress (Nilsson, 1983; Ainslie, 2005; Shi, 2010; Kang 2011). Many studies have shown that shear stress drives SMCs towards a contractile phenotype in 2D or 3D environments (Sterpetti, 1992; Forestermann, 1994; Ueba, 1997; Ainslie, 2005; Ekstrand, 2010), however, there have also been studies on the contrary (Shi, 2010). It is widely understood that more complex, 3D models are needed to understand the true effects of fluid flow on SMCs and how it can be utilized for tissue engineering applications (Stegemann, 2005; Peyton, 2008; Shi,

2011). Kang, et. al. showed that changes in gene expression in response to force in a three-dimensional environment was over two times greater than in a two-dimensional environment (Kang, 2012).

With respect to centritubing, we must be aware of the possible positive and negative consequences of applying these forces to smooth muscle cells. It is expected that the short exposure of forces in centritubing may not have as great an effect on SMC protein expression, orientation, or phenotype as previously described studies using chronic exposure to forces. However, the forces experienced during centritubing are greater than in the studies described, which suggests that cellular changes are likely. It is difficult to predict what these changes might be, however, it is anticipated that centrifugation is a comparable model to centritubing. Unfortunately, the effects of centrifugation on specific cell types, such as SMC, are not characterized in great detail.

2.7 – Centrifugation to Measure Cell Attachment & Detachment Forces

Cell attachment to a substrate plays an important role in cell culture and tissue growth. In centritubing, after cells are pelletted onto the spinning chamber surface they must adhere to the surface so they can form cell-cell adhesions and mature into a cohesive tissue aggregate. It typically takes less than 24 hours for these tissue aggregates to form (Napolitano, 2007), so cells must retain their position upon the spinning chamber wall until then. Surface properties such as material composition, protein adhesion, roughness, and surface tension all play an important role in cell attachment (Absolom, 1979; Warren 1982; Variani, 1986; Absolom, 1988; Eirman, 1989). Integrins play a crucial role in cell attachment to proteins, such as fibronectin that adsorb to substrate surfaces (Boettiger, 2012). Cellular adhesion strength, driven by integrins and the glycocalyx has been shown to increase with time (Boettiger, 2010). Meanwhile, calcium-dependent cell-cell attachment is primarily dominated by cadherin junctions (Takeichi, 1977; Duguay, 2003). To form complex tissue matrices, integrins and cadherins play a role in a network that impacts cell polarity, migration, proliferation, and survival (Weber, 2011). This network includes cellular actin cytoskeleton and connexon junctions between cells, which has been shown to play a significant role in the self-assembly and aggregation of cells (Bao, 2011).

During centritubing, cells must adhere to the spinning chamber as rapidly as possible. Between pelletting and cell attachment during the 15 minute spin, cells must synthesize an adhesive network to achieve sustained attachment to the spinning chamber surface for vertical culture. It is important to investigate methods for attaining strong, rapid cell aggregation and attachment.

There are several methods to measure cell attachment, which require complex equipment, however, centrifugation requires few resources and can provide quantitative measurements of cell attachment, which is why it is used frequently for cell adhesion assays (Hertl, 1984; Garcia, 1997; McClay, 2001; Du, 2011). In general, cells are adhered to a surface, and then the surfaces are inverted and centrifuged under a range of forces, to determine the strength of attachment. For this project the centrifugation cell attachment assay was used because it allows a simple comparison of the magnitude of forces used to attach cells and forces used to detach cells. To quantify cell attachment using centrifugal force, cells can be grown on tissue culture plastic in well plates and inverted in the centrifuge

to apply a detachment force upon the cells. The remaining attached cells can then be counted and the detachment forces can be calculated from RCF (Reyes, 2003).

With respect to centritubing, cell attachment to the seeding surface and one another plays a critical role in the growth of a uniform, cohesive tissue. Subsequently, cells must also be able to detach from the surface once a tissue has formed. In order for the tubular tissue constructs to remain cohesive, the cell-cell attachment strength and the contraction strength of the tissue must be greater than the attachment strength of the tissue to the spinning chamber wall. If the cells are more adhered to the surface than one another, it is hypothesized that the tissue may tear or contract in clumps. These theoretical forces are shown schematically in Figure 10.

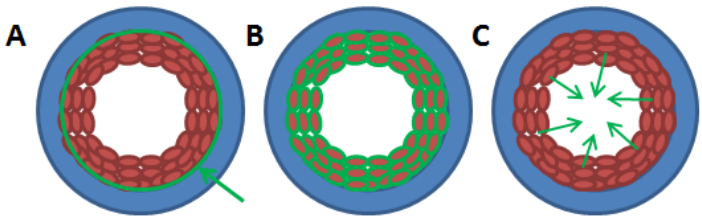

Figure 10 - A schematic representation of cellular factors that affect centritubing success. A) Cell-spinning chamber wall interaction. B) Cell-cell interaction. C) Cell and tissue contraction.

The information presented in this chapter regarding tissue engineered blood vessels, our previous work, and the potential impact of centrifugal and shear forces upon cells, provides background for understanding the rationale and methodology of the experiments necessary to achieve rapid cellular aggregation into a tubular construct by centritubing.

Chapter 3 – Methods

This chapter details the methods used to manufacture the centritubing device and describes protocols used to analyze and optimize the system and its resulting tubular tissue constructs.

3.1 – Laboratory Techniques & Materials

This section describes cell culture and histological techniques used to create and analyze tubular tissue constructs created using centritubing.

Rat Aortic Smooth Muscle Cell Culture

Rat aortic smooth muscle cells (raSMCs), cell line WKY 3M-22 derived from SMCs isolated from 3-month-old adult male Wistar-Kyoto rat aortas by enzymatic digestion were provided by Dr. Thomas Wight (Lemire, 1994; Lemire, 1996). The WKY 3M-22 raSMCs were used unless otherwise specified. Cells were typically cultured in T-150 flasks (BD, 15705-074) or 145x20 mm plates (Greiner Bio-One, CELLSTAR, 639 160) in growth media. Growth media was comprised of Dulbecco's Modified Eagle Medium (Mediatech Inc, 15-017-CV) + 10% Fetal Bovine Serum (PAA Laboratories Inc, A05-201) + 1% Glutamine (Mediatech Inc, 25-015-Cl) + 1% Sodium Pyruvate (Mediatech Inc, 25-000-Cl), 1% Non-essential Amino Acids (Mediatech Inc, 25-025-Cl), and 1% Penicillin-Streptomycin (Mediatech Inc, 30-002-Cl). Cells were cultured in the incubator (Thermo Electron Corporation, HERAcell 150) at 37C and 5% CO_2 until confluent.

Cells were detached from culture surfaces using 0.25% trypsin-EDTA (Mediatech Inc, 25-053-Cl), centrifuged (Beckman Coulter, GS-6R) at 1000 rpm for 5 minutes and then resuspended to the desired concentration.

Sterilization & Disinfection of Custom Culture Apparatus

All unpackaged materials used in contact with media or cells were autoclaved. Materials used in a hood during cell culture that did not come in contact with media or cells were sprayed with 70% ethanol prior to use.

Histology

Tissue samples fixed for trypan blue staining were exposed to 70% ethanol for at least 5 minutes prior to staining. Tissue samples fixed for histology were submerged in 10% neutral buffered formalin for 2 hours, then stored in 70% ethanol until processing and embedding in paraffin. Five-micrometer sections were cut and adhered to Superfrost Plus slides (VWR, West Chester, PA, USA).

Standard stains, such as Hematoxylin & Eosin (H&E) (Richard Allen Scientific, Kalamazoo, MI, USA). The Leica DMIL microscope was used to acquire images of cells in culture and stained tissue samples on slides. To macroscopically observe cell and tissue presence on opaque polycarbonate surfaces, these seeded substrates were soaked in 70% ethanol for 5 minutes, then exposed to trypan blue (0.4% w/v; Cellgro, 25-900-Cl).

3.2 – Spinning Chamber Manufacture

The spinning chamber is the primary component of the centritubing device. The inner wall of this spinning chamber is the cell-seeding surface and the tissue formation substrate. Modifications to the spinning chamber were a central focus of this project. This section details the materials and methods used for the design and validation of the centritubing device.

Initial Manufacturing Method

The preliminary work described in this section was developed as a result of the Major Qualifying Project regarding the invention of centritubing (Burford, 2010). Spinning chambers (Figure 11) were manufactured from a 3/8-inch diameter polycarbonate rod (McMaster-Carr, 8571K13) cut to a length of 3 cm. A lathe was used to drill all holes, because it allowed the drill to be centered relative to the polycarbonate cylinder. The lathe was used to drill a ¼ inch diameter hole down the central axis of the rod, which extends ¾ inch deep into one end. On the other end of the rod, a 1 mm diameter hole was made with a lathe to a depth of ¼ inch. Diameters and lathe depths were measured in inches due to manufacturer specifications. The larger hole of the rod (the spinning chamber) was then covered with silicone glue (Factor 2, A-100-S) to completely seal the chamber. The silicone glue was allowed to cure for 24 hours prior to cell seeding.

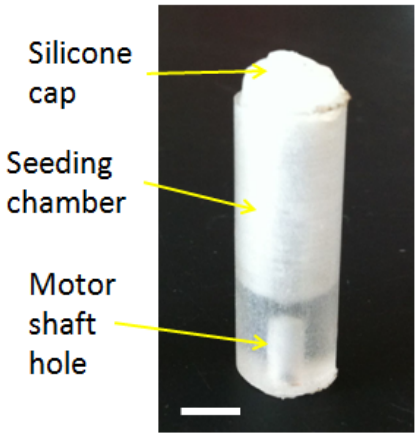

Figure 11 - The polycarbonate spinning chamber for centritubing. Scale bar 5 mm.

Modification to Manufacturing for Smoother Seeding Surface

A modification was made to the drilling of the spinning chamber using the lathe (Figure 12) to produce a smoother seeding surface for cohesive tissue formation. A lathe drilling speed of approximately 0.25 inch per second was used to achieve a smoother surface inside the spinning chamber. The lathe drilling speed refers to the lateral movement (Figure 12C) of the drill bit towards the polycarbonate rod (secured in Figure 12A). Furthermore, the drill bit (secured in Figure 12B) was brushed off to remove debris after each hole was drilled. This became the standard spinning chamber manufacture after Results Section 4.5

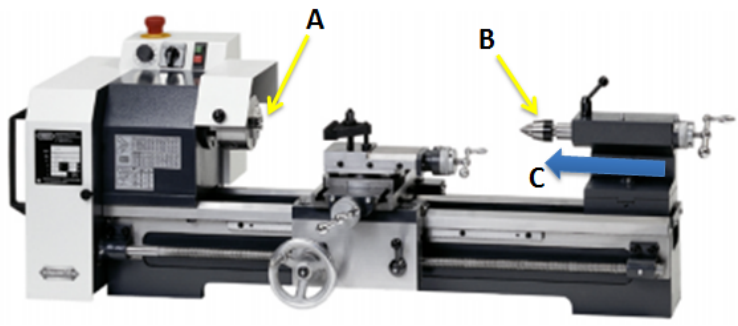

Figure 12 - A lathe was used to manufacture the spinning chamber.
A) Grips hold a polycarbonate rod in place and spin it axially. B) Grips hold a drill bit stationary. C) The stationary drill bit is moved laterally towards the spinning rod. As the drill bit makes contact with the rod it begins to drill the central hole.

3.3 – Centritubing Protocol

Set-Up

Inside a sterile cell culture hood, a 3-volt hobby motor (Radioshack, 273-223) was clamped into a clamp stand horizontally. Alligator clips (Radioshack, 278-1156) were used to create a circuit between the motor and two AA batteries (Radioshack, 23-729) in a 2-battery pack. One alligator clip was detached from the motor to prevent it from running when an experiment was not in progress.

Spinning & Cell Seeding

A 0.4 ml volume of the cell suspension (equal to slightly more than the volume of the spinning chamber) was transferred to a syringe. Using a 27-gauge needle, the cell suspension was injected through the silicone glue cap and into the spinning chamber. Air displacement from within the chamber was accomplished by inserting a second needle through the cap.

Once the spinning chamber was completely filled with the cell suspension, the needles were removed and the chamber was attached to the motor by the hole drilled into the bottom of the polycarbonate rod. This was achieved simply by a tight fit of the hole onto the motor shaft.

Once the chamber was attached, the alligator clips were used to once again create the circuit with the motor. The spinning chamber was then spun axially with the motor shaft as shown in Figure 13. A timer was used to ensure that spinning was conducted for 15 minutes.

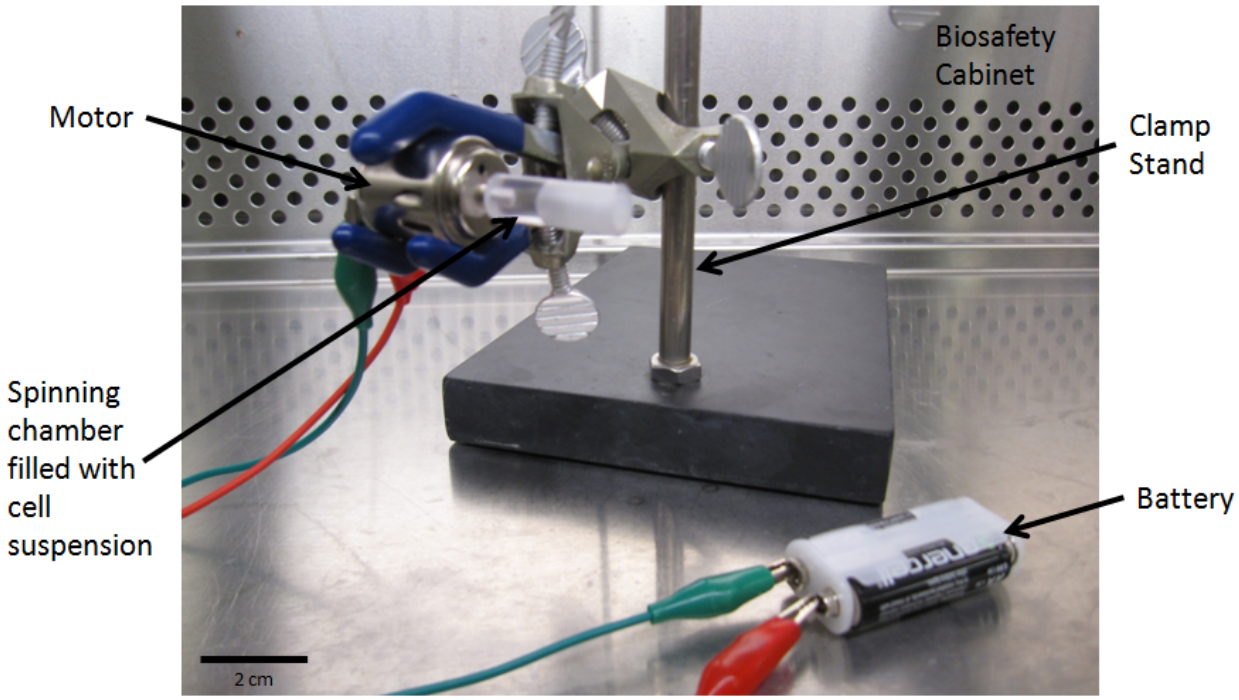

Figure 13 - The entire centritubing system during spinning.

Culture Preparation

The spinning chamber was carefully removed from the motor shaft by hand. A scalpel was used to cut the silicone glue cap from the spinning chamber. The spinning chamber was then inserted into a culture apparatus with media for short-term culture (3 days). The culture system was transferred to an incubator at 37C and 5% CO_2 for the desired culture time.

3.4 – Central Mandrel Manufacture

A central mandrel was developed that could be enveloped by contracting tubular tissue constructs for easier harvesting. The central mandrel was manufactured from a 1/16-inch diameter nylon rod (McMaster-Carr, 8538K11) cut to lengths of ¾-inch. A silicone rubber tube (McMaster-Carr, 51135K11) with OD 1/8-inch and ID 1/16-inch was cut to 5/8-inch. The nylon rod was then slid inside the silicone tube until one end was flush. The central mandrel can be seen in Figure 14.

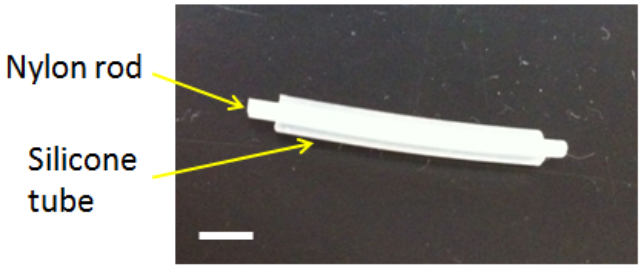

Figure 14 – The central mandrel.
A central mandrel was composed of a nylon rod encompassed by a silicone tube. Scale bar 5 mm.

Modification to Spinning Chamber

A modification was made to the spinning chamber to accommodate the central mandrel and keep it centered within the spinning chamber. Within the larger hole of the spinning chamber, a 1/8-inch diameter hole was drilled 1/8-inch deep. The central mandrel could then be inserted into this hole (using the exposed nylon end) to keep it centered within the chamber (Figure 15).

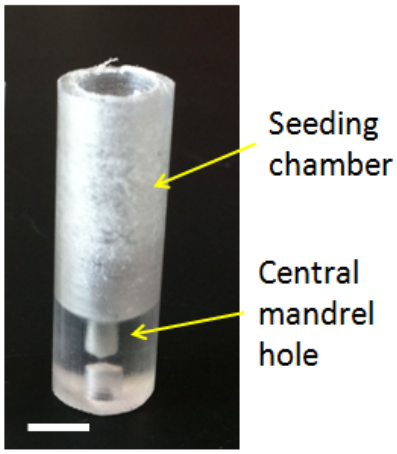

Figure 15 - The spinning chamber with a hole drilled for the central mandrel.
Scale bar 5 mm.

3.5 – Culture Apparatus Manufacture

The purpose of the culture apparatus is to hold multiple spinning chambers and media to support the growth of tubular tissue constructs after centritubing. Three different culture apparatus designs were tested.

Conical Culture Apparatus

The conical culture apparatus was made by combining a sterile 50 mL conical tube, a sterile T-75 gas exchange screw top, and a sterile cryovial. A hole was drilled into the bottom of the conical flask after which the top of the T-75 flask was fitted and sealed to the hole. Incorporation of the flask filter top was performed to allow gas to diffuse through the cap while preventing contaminating agents from

entering the system. Lastly, a sterile cryovial with a 3/8 inch inner diameter was glued to the underside of the conical tube cap. This piece was intended to hold the spinning chamber in place during culture.

Alternatively, the conical culture apparatus can be created without the media exchange port and syringe to minimize chances for contamination. Instead of attaching the T-75 cap directly to the conical tube, the entire neck of the T-75 can be cut off and attached to the apparatus. This allows the vented cap to screw on and off for easy addition and removal of media. Furthermore, sterile silicone glue can be used to attach the cryovial to the cap and to attach the vented top to the conical tube. It is important to note that the conical culture apparatus should be used with the conical tube resting on its cap and the bottom end facing up as seen in Figure 16 below. Extra precautions must be taken to avoid contamination because the materials used cannot be sterilized by autoclave, which means the culture apparatus can only be assembled and used once before being discarded.

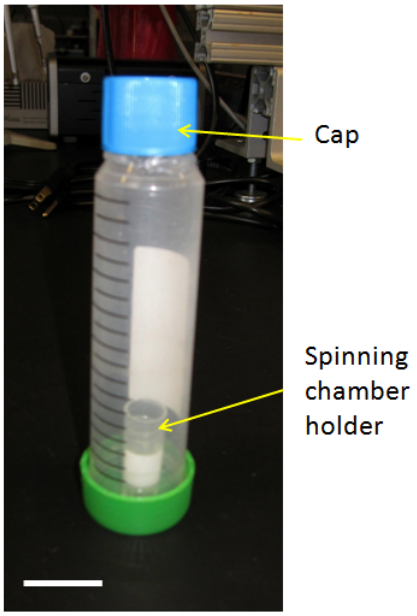

Figure 16 - A photograph of the conical culture apparatus. Scale bar 2 cm.

Box Culture Apparatus

The box culture apparatus was used after leaking occurred with the conical culture apparatus. Polycarbonate sheets (¼ inch thick; McMaster-Carr, 8574K55) were cut to create three 3x8 inch rectangles. These were glued together using Devcon 5-Minute Epoxy (Lowe's, 74809) and used for the bottom, sides, and top of the chamber. The remaining polycarbonate material was cut to fit for the ends and set in place with the epoxy. A ¼ inch drill bit was used to drill 3/8 inch deep holes in the inside bottom of the box—for holding spinning chambers during culture. Silicone glue was used to seal the edges.

PDMS Culture Disk System

The purpose of the PDMS culture disk system was to replace the conical culture apparatus and box culture apparatus as a spinning chamber holder for culture that will be sterile, not leak, and allow

for spinning chambers to be attached with minimal difficulty. A PDMS mold was created in the bottom of a 100mm non-treated tissue culture dish. Six pieces of the same polycarbonate rod used for the spinning chamber were cut to a length of ¼-inch and glued equidistant in a circle to the bottom of the culture dish. A small plunger from a 1-ml syringe was also glued in the center of the circle. Polydimethylsiloxane (PDMS) (Ellsworth Adhesives, 184 SIL ELAST KIT 3.9KG), Sylgard 184 Silicone Elastomer Base was mixed with Sylgard 184 Silicone Elastomer Curing Agent at a 10:1 weight ratio using a bowl and stirring stick. The mix was then poured into the mold until the polycarbonate disks were completely covered. The cast was degassed until bubbles were removed. Then the cast was transferred to an oven at 90C and incubated for 2 hours to cure. The PDMS cast was cut out in a circle or polygon around the polycarbonate disks using a scalpel and the cast was peeled from the dish. The assembled spinning chamber holder (Figure 17) was autoclaved before use.

A handle was created using a sterile, disposable syringe plunger inserted into the central hole of the PDMS disk. The excess underneath material was trimmed to be flush with the underside of the disk. This was used as a handle to easily move the holder.

For culture, spinning chambers were inserted into the indents created from the polycarbonate disks in the mold and the holder was transferred to a 200 ml beaker (Figure 17). This was then filled with 150 ml of media to adequately submerge all spinning chambers. A sterile tissue culture dish top was used to enclose the system.

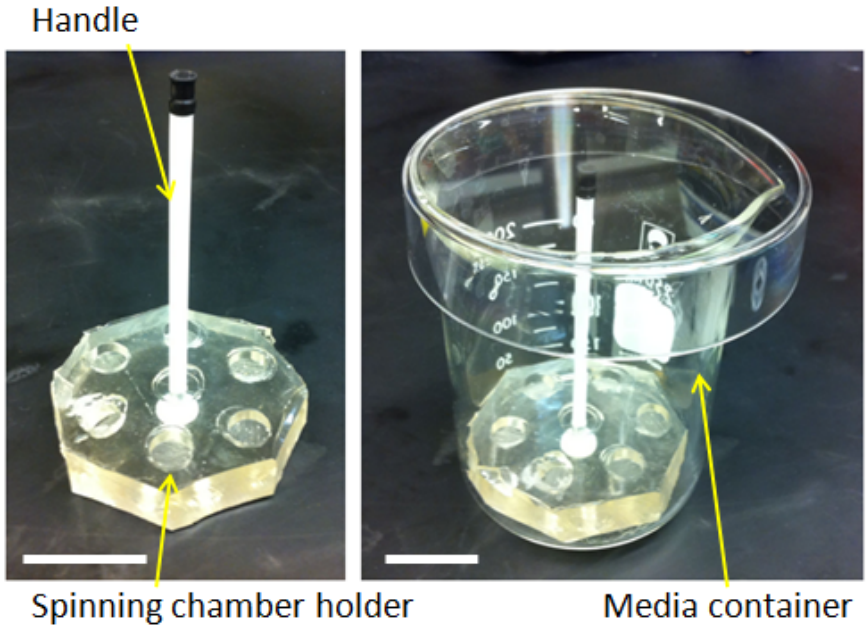

Figure 17 - The PDMS disk culture system.
The PDMS disk (A) culture system can fit within a standard laboratory beaker for culture (B). Scale bars 2 cm.

3.6 – Tissue Tube Harvesting

Tissue tubes were harvested from spinning chambers by first aspirating media from the culture apparatus. Next spinning chambers were removed from the culture apparatus and placed in a Petri dish. Forceps were used to carefully lift the central mandrel from within the spinning chamber, being careful to avoid hitting the mandrel against the sides of the chamber wall. Tissue tubes were left on the central mandrel for fixation and processing for histology.

3.7 – Tissue Thickness & Length Measurements

A machine vision system (model 630; DVT Corporation, Atlanta, Ga., USA) was used to take images and measure the thickness of the tissue tube samples contracted around the central mandrels. To acquire measurements, digital sensors were used that identify a sharp change in white to black on the images (representing the sides or ends of the tissue tubes) using edge detection software (Framework 2.4.6; DVT). Sensors at these locations were used to measure the distance (in pixels) between one another. These measurements were then converted to millimeters using the known value of the central mandrel outer diameter (3.25 mm). The wall thickness could then be calculated from the total thickness of the tube by subtracting the central mandrel thickness value and dividing by 2.

3.8 – Cell Attachment & Viability Studies

To understand and control the cellular forces involved in centritubing, several cell attachment studies were performed.

Cell-Substrate Attachment Strength

Cell attachment to the spinning chamber plays an important role in centritubing, making it crucial to quantify the magnitude of force required to detach cells. Knowing how many cells remain attached after a specific detachment force is applied can provide information regarding how strongly the cells are adhered to the surface. Preliminary cell-substrate attachment studies in this project were based on a common technique utilizing a centrifuge to control detachment force (Hertl, 1984). Cells were attached to culture plates by gravity (1 xg force) or a centrifuge at a variety of forces. To quantify solely the effect of attachment force on cell attachment, gentle media flow was applied to detach cells. To quantify the strength of attachment and detachment, cells were detached by inverting culture plates in the centrifuge or in the cell culture hood (175.3 xg force versus 1 xg force).

Cell-Cell Aggregation

In centritubing, cells aggregate and attach to one another during and after the spinning process to form a cohesive tubular construct. It is important to provide a means for rapid cell-cell attachments to form during the spinning process. Augmenting trypsin with a 2mM concentration of $CaCl_2$ has been shown to improve rapid aggregation over standard trypsin-EDTA treatments (Takeichi, 1977). To observe the effects of trypsin-EDTA, trypsin, and trypsin-CaCl2, cells were trypsinized and seeded on culture plastic or agarose-coated plastic. To make the agarose-coated surface, SeaKem LE Agarose (Lonza, 50000) powder was mixed with DMEM at 2% of the final weight. The agarose was autoclaved on the liquid cycle to sterilize. Once agarose was in a liquid state, it was pipetted into the culture well and

transferred to a refrigerator to gel (5 minutes). Cells were observed under light microscopy at various time points.

Alamar Blue

Alamar Blue (Invitrogen, DAL1025) was used to measure metabolic activity of cells in Results Section 5.2. Dye was added to wells at 10% (100 µm). Fluorescence was read at 544 nm excitation and 590 nm emission wavelengths at 7 and 10 hours after cell seeding as instructed in manufacturer manual.

Chapter 4 – Centritubing Results

Centritubing was designed as a method to rapidly assemble tubular tissue constructs entirely from cells. In our previous work, a proof of concept for centritubing was developed, but the system did not consistently produce a tubular tissue construct. The objectives of the experiments reported in this chapter were to utilize centritubing to achieve cellular aggregation into a tube with a set lumen diameter and also to improve the success rate of tube formation in centritubing.

To accomplish these goals, several design considerations were identified from our previous work (Burford, 2010) and throughout the subsequent experiments. These design considerations were observed as complications that may have resulted in tissue tube failure:

- Bacterial contamination
- Incomplete injection of cell suspension
- Wobbling of spinning chamber during spin
- Excessive motor vibration during spin
- Silicone cap detachment
- Cap leaking during spin
- Rough inner surface in the cylindrical seeding chambers
- Disruption of seeded cells prior to aggregation
- Culture apparatus leaking
- Difficulty harvesting tubes from the spinning chamber after culture
- Uncontrolled tissue contraction

We hypothesize that the success rate of tube formation by centritubing will be increased by addressing these design considerations and implementing design modification to solve them.

4.1 – Observing the Potential Causes of Tube Formation Inconsistency in the Centritubing System

The objective of this experiment was to identify any problems in the methods of centritubing that may lead to inconsistency in tube formation. To accomplish this, six tubes (batch 1.003, referring to notebook and page number) were created by centritubing for 15 minutes, each with $25x10^6$ raSMCs. Spinning chambers were then cultured in conical culture apparatuses for three days.

All but one sample had successful, smooth, and uniform spins while they were spinning on the motor. A successful spin was defined as a spinning chamber centered on the motor shaft correctly, with minimal or no wobbling of the spinning chamber and little vibration of the motor during spinning. Although the spinning appeared successful, the motor became unclamped due to vibration during spinning for two of the samples. In two other cases, the silicone glue cap used to keep the cells in the chamber during spinning came loose from the spinning chamber. This was likely caused by a lack of adequate surface area contact between the glue and the chamber. These spinning chambers were replaced and new cell suspensions were added.

Culturing tubes using conical culture apparatuses resulted in dissociated tissue aggregates. This tube failure was likely caused because each chamber leaked through a crack in the bottom lid. The crack may have been caused by excessive tightening of the cap, the temperature of 37C softening the plastic material of the cap to an extent that it was more malleable, or a combination of both.

The primary concern from this experiment was the leaking of the conical culture apparatus. Furthermore these culture apparatuses are not reusable and are not sterile after manufacture.

4.2 – Box Culture System Failure

The objective of this experiment was to test the practicality of a redesigned box culture system (described in Methods section 3.5). To test this change, five tubes (batch 1.005) were centritubed for 15 minutes, each with $25x10^6$ raSMCs. Spinning chambers were then cultured together in the box culture apparatus.

All samples had successful spins, but no tissue tubes were formed, only dissociated clumps of tissue. The box culture apparatus system did not leak, however it was difficult to attach spinning chambers into their slots within the box. The slots were designed to be tight fit, but they may have been too tight and caused too much jostling and bumping to attach the spinning chambers securely. This may have disrupted the seeded cells in the spinning chambers.

The motor vibrations from spinning during centritubing caused the entire surface of the hood to visibly shake. It is possible that this vibration disrupted cells that had already been seeded onto previously centritubed spinning chambers still in the hood. This disruption could potentially have resulted in failed tube formation. It is also possible that the complications associated with the box culture system caused disruption of seeded cells.

4.3 – Proof of Concept for Central Mandrel

In our previous work, tubular tissue constructs were created by centritubing, but were difficult to harvest from the spinning chamber and had unconstrained circumferential contraction. This circumferential contraction resulted in variable lumen diameters between the tubular tissue constructs. In Chapter 2, it was shown that centritubed tissue contracted during culture and remained attached to the spinning chamber wall at one location. After contraction, the tubular tissue construct was removed gently by sliding forceps through the lumen, but the tissue was still fragile and structurally weak (Burford, 2010).

To provide a substrate for the contraction of the tubular tissue construct during culture and improve the ease of removal from the spinning chamber after culture, a central mandrel was implemented. The central mandrel was designed to be a non-adhesive rod centered within the spinning chamber that would provide a substrate for the tissue tube to envelope as it contracted from the chamber wall, as shown in Figure 18. This mandrel would act as a contraction and remodeling substrate with a set lumen diameter, which would also allow for less disruptive harvesting of the tubular tissue constructs.

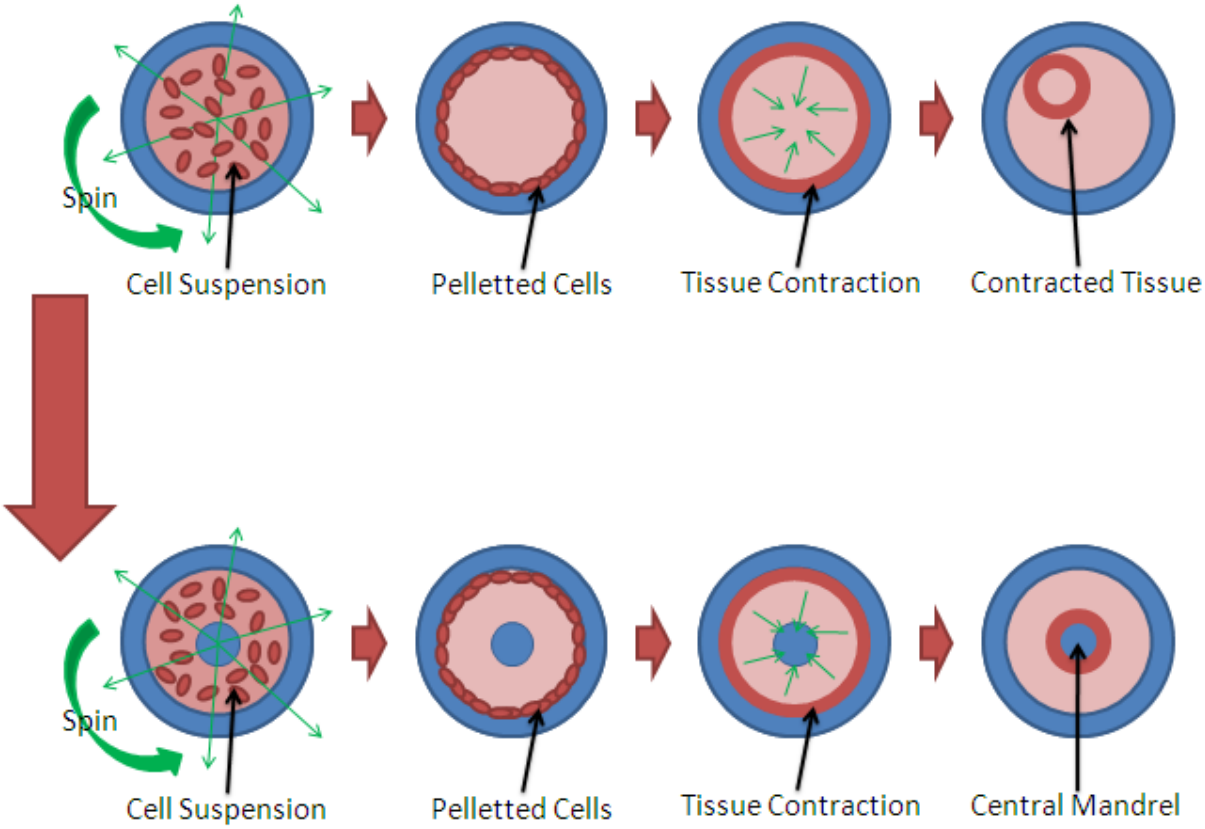

Figure 18 - A schematic representation of a tissue tube contraction during centritubing. With no substrate to contract upon (top) versus contracting around the central mandrel (bottom).

To accommodate this central mandrel, an additional hole was drilled inside the bottom of the spinning chamber and the inner nylon rod (the structural component of the central mandrel) was fit into the hole. Silicone tubing was used around the nylon rod as the tissue contraction substrate due to its non-adhesive properties. The intent was for the central mandrel to stay centered during spinning, while also allowing for easy removal after culture without compromising spinning dynamics, seeding effectiveness, or tissue formation. Previous work in our lab showed success in growing tissue on silicone tubing (Doshi, 2009).

The purpose of this experiment was to test a proof of concept that the presence of the central mandrel would not prevent cells from pelletting with complete coverage on the spinning chamber wall. To test this, $25x10^6$ raSMCs were centritubed in one spinning chamber with no central mandrel and one spinning chamber with a central mandrel. Post-centritubing, the media in the spinning chamber was removed and the cells pelletted to the spinning chamber wall were fixed and stained with 70% ethanol for 5 minutes and trypan blue for 5 minutes, then rinsed with PBS.

The two spinning chambers that were centritubed using a central mandrel both showed complete cell coverage based on trypan blue staining, which has been used as an indicator of cell

presence previously (Burford, 2010). Trypan blue-stained cells seeded within the chambers are shown in Figure 19. With complete cell coverage in the presence of a central mandrel, the next step was to test the central mandrel's ability to control tubular tissue contraction.

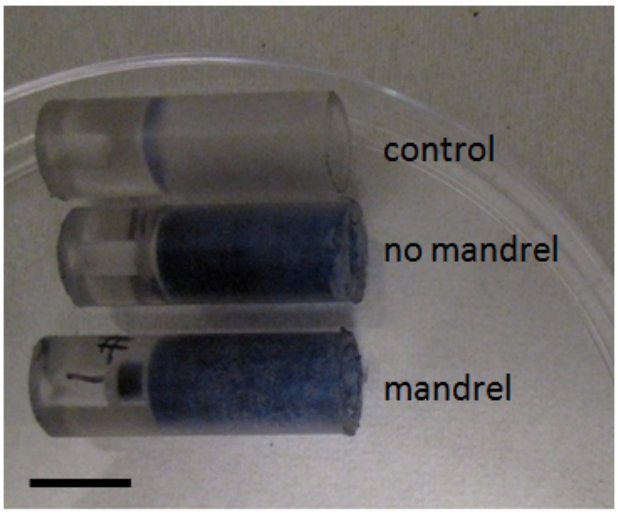

Figure 19 – Cell coverage with and without central mandrel.
Cell seeding was shown macroscopically by a trypan blue stain. Scale bar 1 cm.

4.4 –Controlled Tissue Contraction & Culture

In section 4.3, we showed that spinning chambers centritubed both with and without a central mandrel exhibited complete cell seeding. The primary objective of this experiment was to determine the success rate of the central mandrel as a contraction substrate for tissue tubes in culture. Two secondary objectives were to observe the potential of the central mandrel for easier and less disruptive harvesting than clamping the tissue constructs with forceps and also to test a new culture system.

The new culture system (shown in Figure 20) was designed to avoid leaking, use less media, and allow insertion of spinning chambers more gently. A culture disk was created from PDMS with holes the same diameter as the spinning chambers. The elastic nature of PDMS allowed for much easier insertion of spinning chambers with no jostling or disruption. The manufacture of the culture apparatus is described in Methods section 3.5.

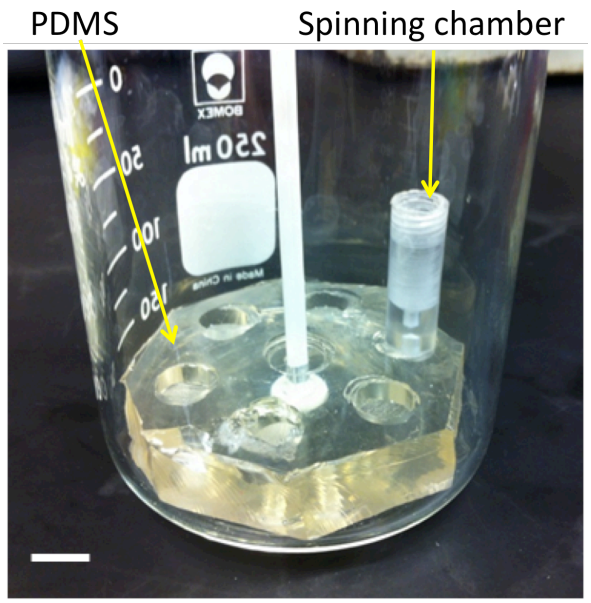

Figure 20 - New PDMS Disk Culture System. Scale bar 1 cm.

To test these objectives, $22x10^6$ raSMCs were centritubed into three spinning chambers (batch 1.021). Previously, $25x10^6$ raSMCs were used for centritubing, but previous experiments suggested that fewer cells could be used for successful tube formation (section 5.1). All spinning chambers showed a successful spin as defined previously. After each sample was spun, the cap was removed and the spinning chamber was moved to a separate sterile hood to avoid vibration caused by the motor in the centritubing system, as observed previously. After all samples were spun they were transferred to the new PDMS disk. This culture disk was then inserted into a 500 ml glass beaker, which was filled with media just to cover the spinning chambers and capped for culture.

After observing tissue clumps forming rather than cohesive tubes in sections 4.1 and 4.2, we wanted to identify at which point in time the tissue begins to contract. We hypothesized that this might help to identify a cause of this breaking up of the tissue. To observe tissue formation progress at 24, 48, and 72 hours, the culture system was removed from the incubator and media was aspirated from the culture apparatus. The culture disk was removed from the culture apparatus and samples were observed macroscopically through the spinning chamber walls. After observations were noted, the culture disk was placed back into the culture apparatus and media was replaced.

After one day in culture, all samples showed signs of some tissue contraction; however, it was unclear whether they were contracting as a tube or as independent aggregates of tissue. After two days in culture, samples showed signs of tubular contraction. Two samples had contracted fully around the central mandrel, but all samples appeared to still be in contact with the spinning chamber wall, as shown in Figure 21.

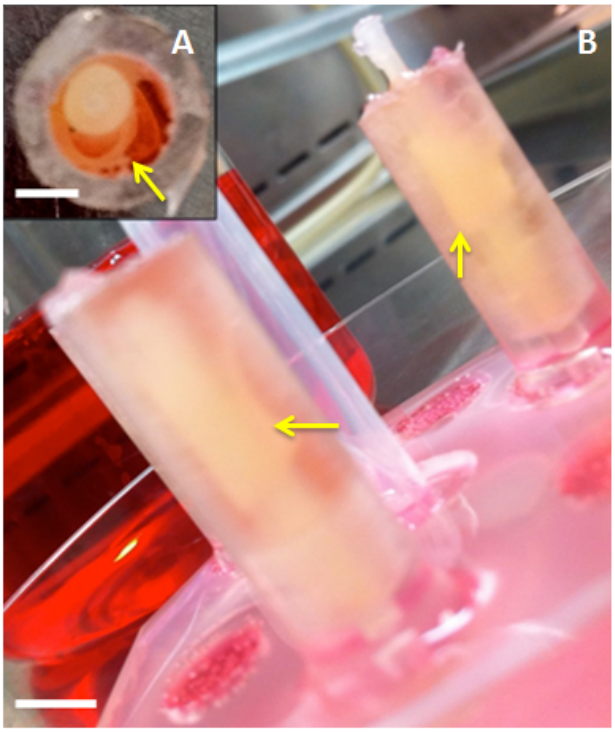

Figure 21 - Centritubing results after two days in culture. Samples showed signs of tubular tissue contraction around the central mandrel (shown in A & B). Tissue was still attached to the spinning chamber wall as well (shown in A). Arrows show tissue formation. Scale bars 3 mm.

After three days in culture, samples appeared to be completely contracted around the central mandrels. The central mandrels were removed from spinning chambers by grabbing the top end with forceps. One sample was harvested easily and remained on the central mandrel in PBS. One sample was almost removed, but slid off the central mandrel during harvest. The tissue tube was recovered with forceps and moved to PBS. The final sample was still somewhat attached to the spinning chamber wall and remained in the spinning chamber when the central mandrel was pulled out. Each sample is shown in Figure 22.

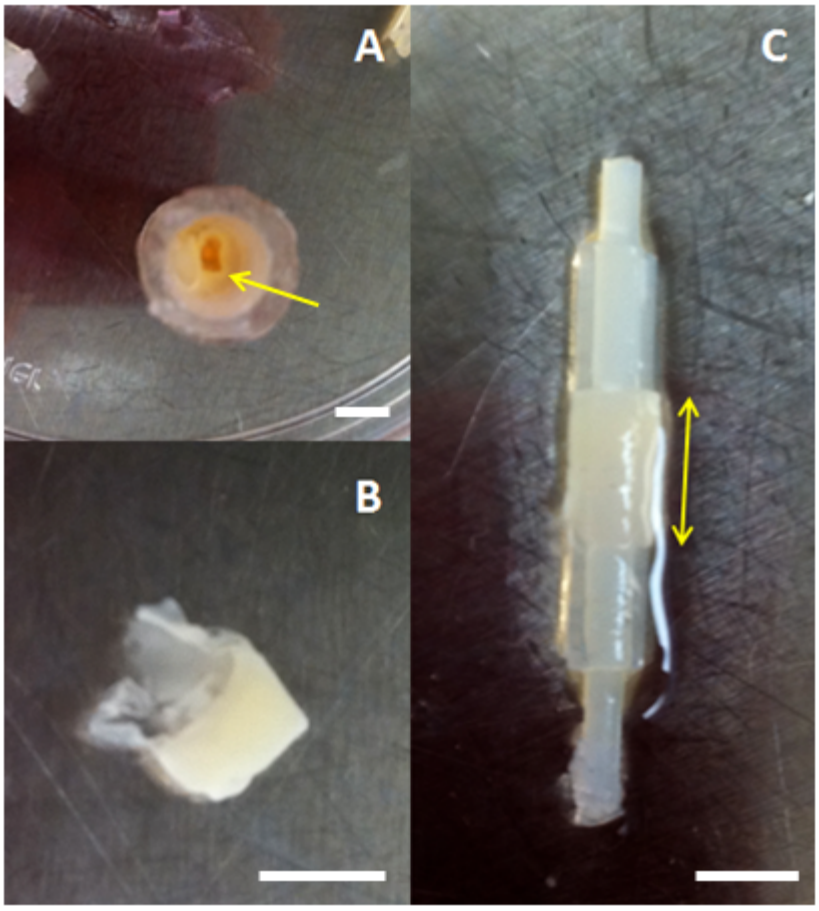

Figure 22 – Tissue tubes formed from centritubing after 3 days in culture. Tubes were not consistent in length (shown in B & C) and did not adhere strongly to the central mandrel (shown in A). Arrow displays length of tissue on central mandrel. Scale bars 3 mm.

These same methods were repeated for batch 1.024 (n = 2), but samples exhibited no successful tube formation. Unfortunately, tissue contraction was non-uniform and irregular. The tissue did not appear to be cohesive and contracted in patches and clumps, as shown in Figure 23. It was hypothesized that this dissociation was caused by roughness and non-uniformity of the spinning chamber walls.

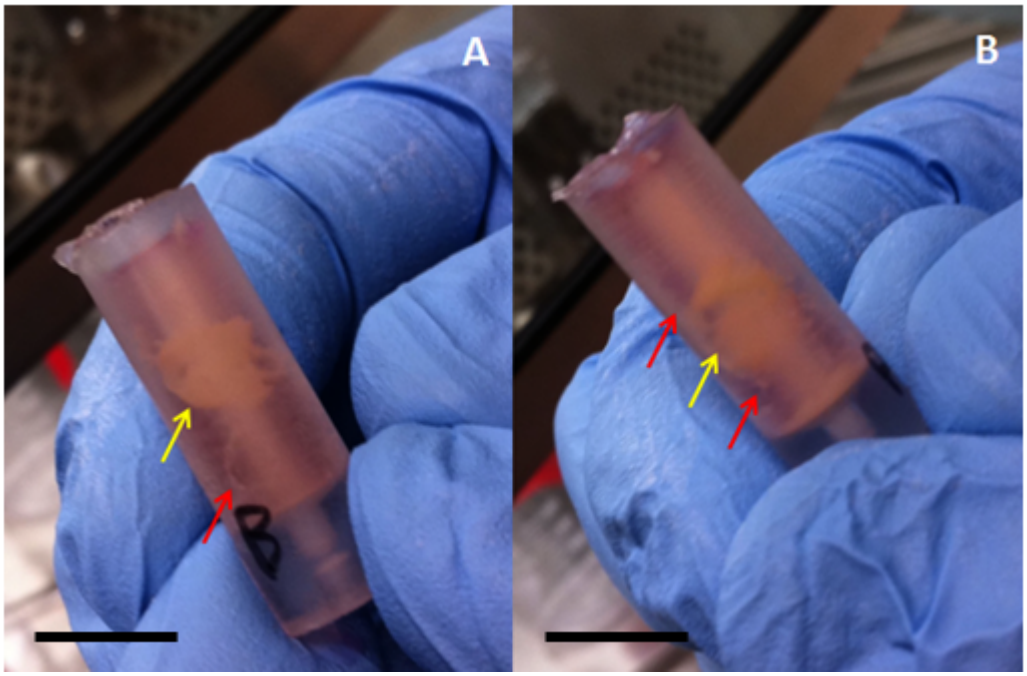

Figure 23 – Three day poor tissue formation.
After three days in culture post-centritubing, tissue had contracted in patches and clumps, not a uniform cylinder. A & B are two different samples showing similar contraction. Yellow arrows show irregular contracted tissue, red arrows show potential areas of rough wall inside spinning chambers. Scale bars 1 cm.

4.5 – Manufacturing a Smoother Seeding Surface for Cohesive and Uniform Tissue Formation

After observing the non-uniformity and roughness of the spinning chamber wall (the seeding surface), slight changes were made to the manufacture of the device. Previously, during the drilling of the spinning chamber, the lathe was moved at no set speed, simply until the chamber was drilled to the correct depth. Furthermore, the drill bit for lathing was not cleaned in between each spinning chamber manufacture. For this test, one spinning chamber (batch 1.025 A) was used that had been created by lathing at a slower rate to reach the desired spinning chamber depth and the drill bit was cleaned. Two spinning chambers (batch 1.025 B, C) were used that had been lathed at a faster rate and the drill bit was not cleaned. A macroscopic comparison of these resulting surfaces is shown in Figure 24.

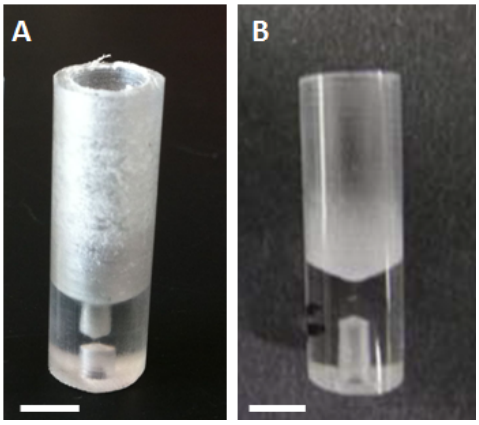

Figure 24 - A macroscopic comparison of spinning chamber wall roughness. Image A was manufactured without consideration for lathe speed or cleaning. Image B was manufactured by the new smoother method. Scale bars 5 mm.

The remainder of the centritubing method was consistent with the experiments in section 4.4. Twenty million raSMCs were centritubed into the three spinning chambers described above.

After culture for three days, the spinning chamber with the smoother surface showed signs of tissue tube formation and contraction around the central mandrel. The central mandrel was removed using forceps and a cohesive tissue tube was observed (Figure 25A). On the other hand, the spinning chambers with a rough wall showed signs of non-uniform tissue contraction in clumps (Figure 25B).

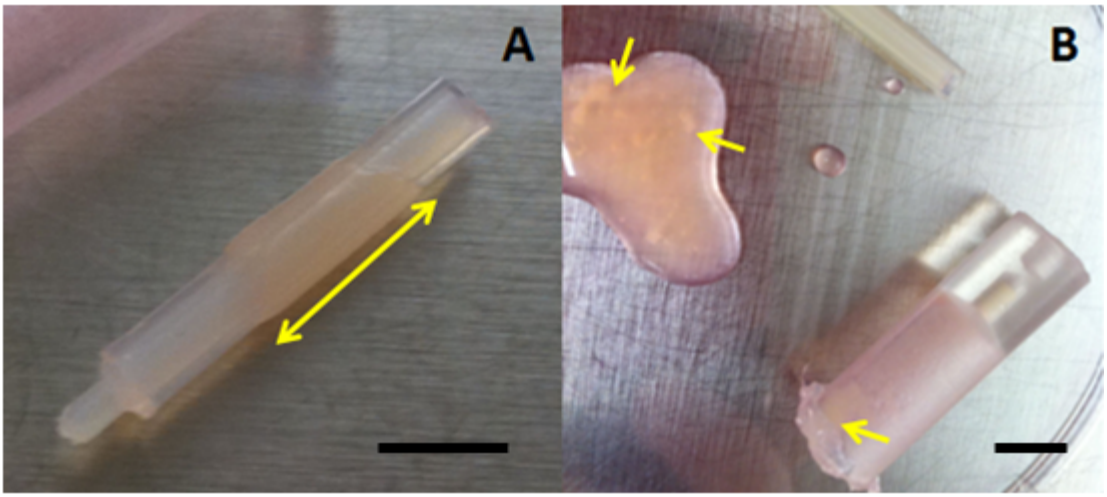

Figure 25 – Tube formation in smooth versus rough surfaces.
A) After three days of culture, tissue contracted around the central mandrel in the smooth spinning chamber. Arrow displays length of tube. B) Cells contracted in non-cohesive clumps in the rough spinning chamber. Arrows highlight several dissociated tissue clumps. Scale bars 6 mm.

Ensuring that spinning chambers had holes that were centered helped to avoid wobbling during spinning, drilling a smoother seeding surface in the spinning chamber resulted in tubular tissue construct formation, and using smaller gauge needles for cell injection avoided leaking through the cap during spinning.

4.6 – Observing Repeatability in Tube Formation With a Smoother Seeding Surface

The results from section 4.5 suggested that slower drilling of spinning chambers and cleaning the drill bit frequently created a smoother surface that better facilitated cohesive tissue tube formation. To test how this change affected tube formation success, additional batches (1.026, 1.028, 1.030) were created using spinning chambers manufactured to have a smoother surface (n = 12 total).

Batch 1.026

Five spinning chambers were prepared and $20x10^6$ raSMCs were injected and centritubed in each. After three days in culture, all samples showed signs of cohesive tissue formation. Three samples had formed complete tubes that contracted around the central mandrel (two are shown in Figure 26). Two samples appear to have formed half-tubes, split down the long axis (shown in Figure 27). We hypothesize that, after the tissue already contracted around the central mandrel to form a tube, it continued to contract circumferentially and eventually ripped the tissue at a weak point.

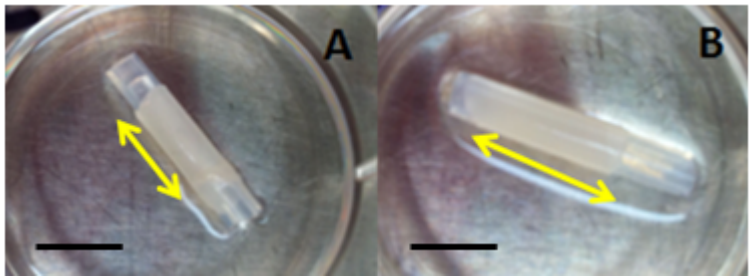

Figure 26 – Uniform tube formation.
A) Sample 1.026-C and B) sample 1.026-E showed uniform and cohesive tissue tube formation and contraction around the central mandrel after three days in culture. Arrows indicate length of tissue tubes. Scale bars 6 mm.

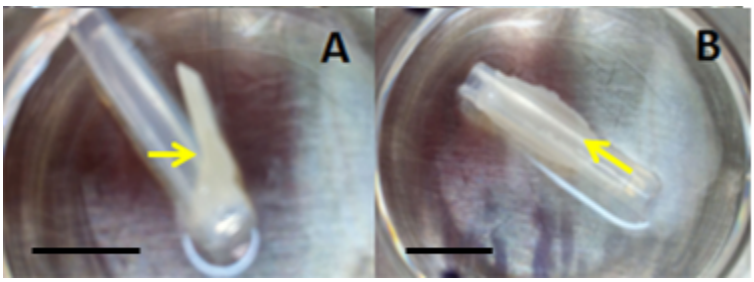

Figure 27 – Split tube formation.
A) Sample 1.026-D and B) sample 1.026-A showed cohesive tissue formation and contraction onto the central mandrel after three days in culture, however, these did not remain (or never formed) complete tubes. Arrows indicate where tissue tubes split. Scale bars 6 mm.

Hematoxylin & eosin (H&E) stains were performed on all samples. This stain showed dense tissue formation, but also an inconsistency in tissue thickness within a sample (Figure 28).

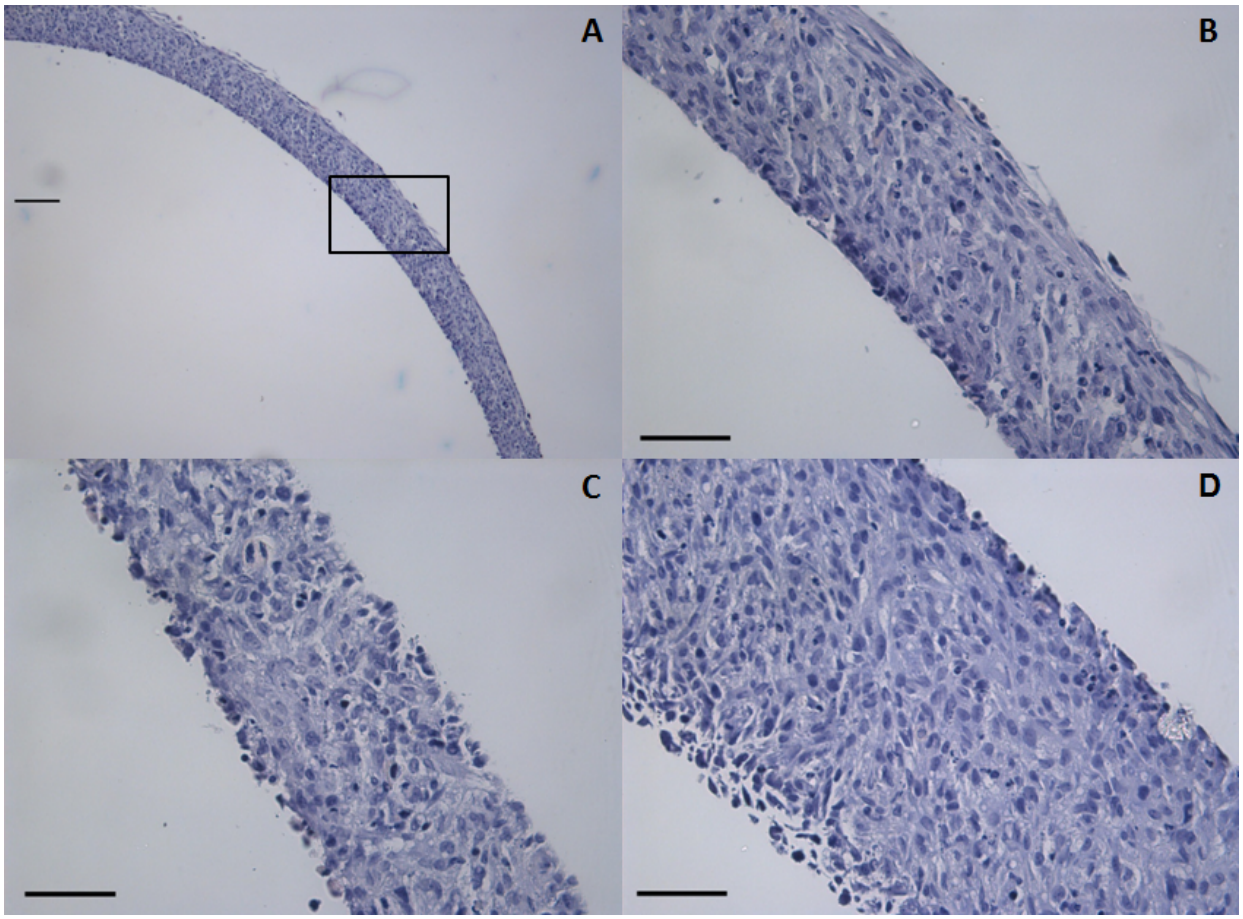

Figure 28 - H&E staining of tissue tubes. Tubes showed high cell density (A, B), but also inconsistency in wall thickness (C, D). Scale bars 100 µm.

Batch 1.028

For this batch, $20x10^6$ raSMCs were centritubed in four smoother surface spinning chambers. After three days in culture, all samples showed signs of cohesive tissue formation. Two samples appeared to be complete tubes that contracted around the central mandrel. These two were harvested, but the other two showed no signs of failure and a layer of tissue appeared to have formed upon the wall, so they were left in culture to complete contraction off the spinning chamber wall. During harvest, sample 1.028-A touched the spinning chamber wall and slid off the central mandrel prematurely, while sample 1.028-B was still somewhat attached to spinning chamber wall, causing it to slide of the central mandrel and break into pieces (shown in Figure 29). Figure 29 provides evidence that these tubes show little structural strength or integrity after 3 days in culture.

Due to the poor harvesting results of the three-day tissue tubes, the remaining two samples were left in culture until they appeared to be completely detached from the spinning chamber walls. After ten days, they were harvested successfully as the tissue tubes had completed contraction around the central mandrel. Sample 1.028-D did slide off the central mandrel after harvesting (shown in Figure 30).

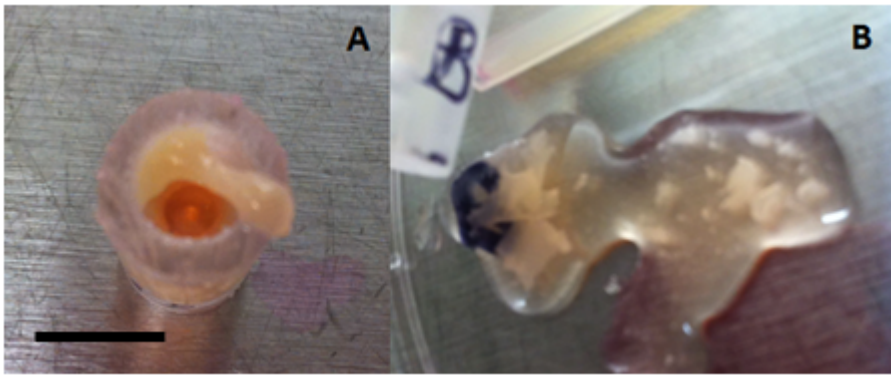

Figure 29 – Three day tissue tubes destroyed during harvest. Tubes slid off the central mandrel during harvest and were destroyed because they were still slightly adhered to the spinning chamber wall. A) slid off central mandrel during harvest while B) slid off and fell apart. Scale bar 6 mm.

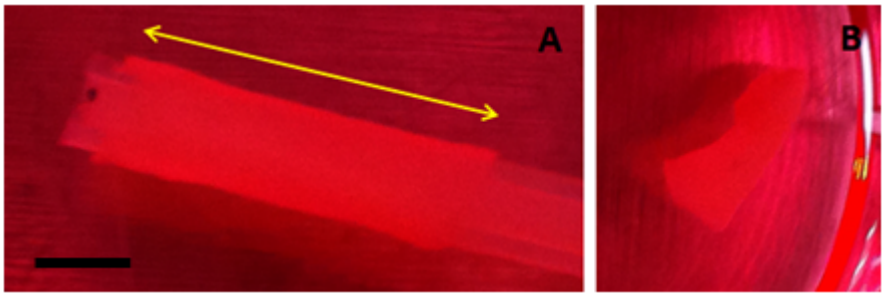

Figure 30 - Tissue tubes that were cultured for ten days. Tubes did aggregate after three days in culture, but appeared to be only partially contracted off the wall. They remained in culture until it was clear they were no longer attached to the wall to avoid destruction during harvest. A) remained on the central mandrel after harvest, but B) slid off shortly after the central mandrel was removed from the spinning chamber. Scale bar 3 mm.

Batch 1.030

For this batch, $20x10^6$ raSMCs were centritubed in three smoother surface spinning chambers After three days in culture, only one sample formed a complete tissue tube (Figure 31A). Results suggested that motor vibration or problems with the central mandrel hitting the cell-seeded spinning chamber wall before culture may have caused cells to form tissue in patches (Figure 31B & C) rather than in a complete tubular sheet.

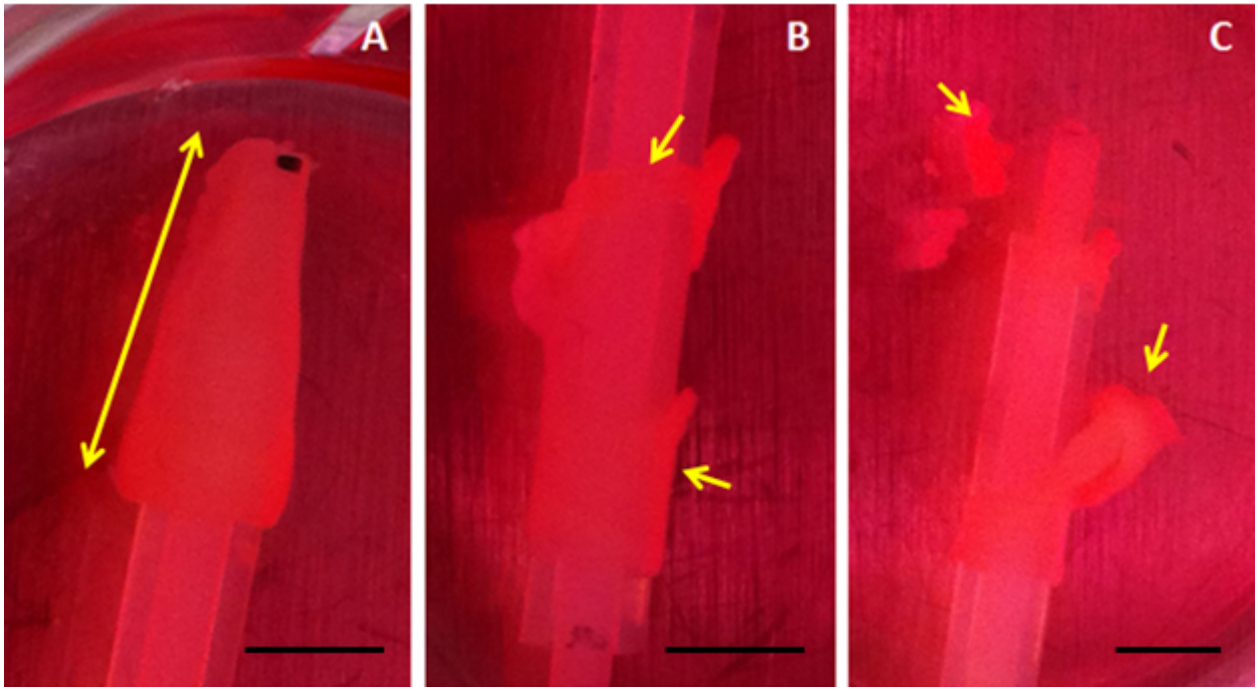

Figure 31 – Three day tubes on smooth seeding surface. After three days in culture, one samples formed a complete tissue tube contracted around the central mandrel A), while two others contracted around the central mandrel in incomplete clumps and patches B) & C). Arrows indicate the formed tube and tissue clumps. Scale bars 3 mm.

4.7 – Centritubing Repeatability Quantitative Analysis

To quantify the success rate of tube formation by the centritubing method, the results from sections 4.4 – 4.6 have been compiled in Table 1. The tissue contracted column refers to samples where tissue formed and contracted onto the central mandrel. The tubular shape formed column refers to the samples that actually formed tube-like shapes around the central mandrel. It does not include tissues that contracted onto the central mandrel in clumps and aggregates. It is important to note that the half-tubes described in batch 1.026 were considered successful tube formation for this analysis, because they appear to have fully formed tubes and then split afterwards. They did contract upon the central mandrel in a tube-like formation.

Table 1 - Centritubing Success Rate Compiled from Sections 4.4 - 4.6.

Batch (section)	Samples Spun	Tissue Contracted	Tubular Shape Formed
1.021	3	3	3
1.024	2	2	0
1.025	3	3	1
1.026	5	5	5
1.028	4	4	4
1.030	3	3	1
Total	**20**	**20**	**14**
Rate	**100%**	**100%**	**70%**

Based on the centritubing repeatability results, it is evident that if there are few disturbances during spinning, tissue will form on the spinning chamber wall and will begin to contract. However, the tissue may not always contract onto the central mandrel in a tubular shape as intended in every circumstance. In many cases, it is hypothesized that the roughness of the spinning chamber wall or scraping of cells off the wall by the central mandrel inhibited uniform and completely cohesive tubular formation. In experiments where a smoother spinning chamber wall was used and tissue did form after three days in culture, there is a high success rate of 85% (11/13) for tubular tissue formation.

Along with investigating the overall success rate of the centritubing method, it was also important to quantify the consistency in the resulting tissue tubes. To do this, tube length and thickness were measured using a machine vision system called DVT, described in the Methods (section 3.7). DVT images used to take these measurements are shown in Figure 32. These measurements provided an average wall thickness of 358 ± 46 μm.

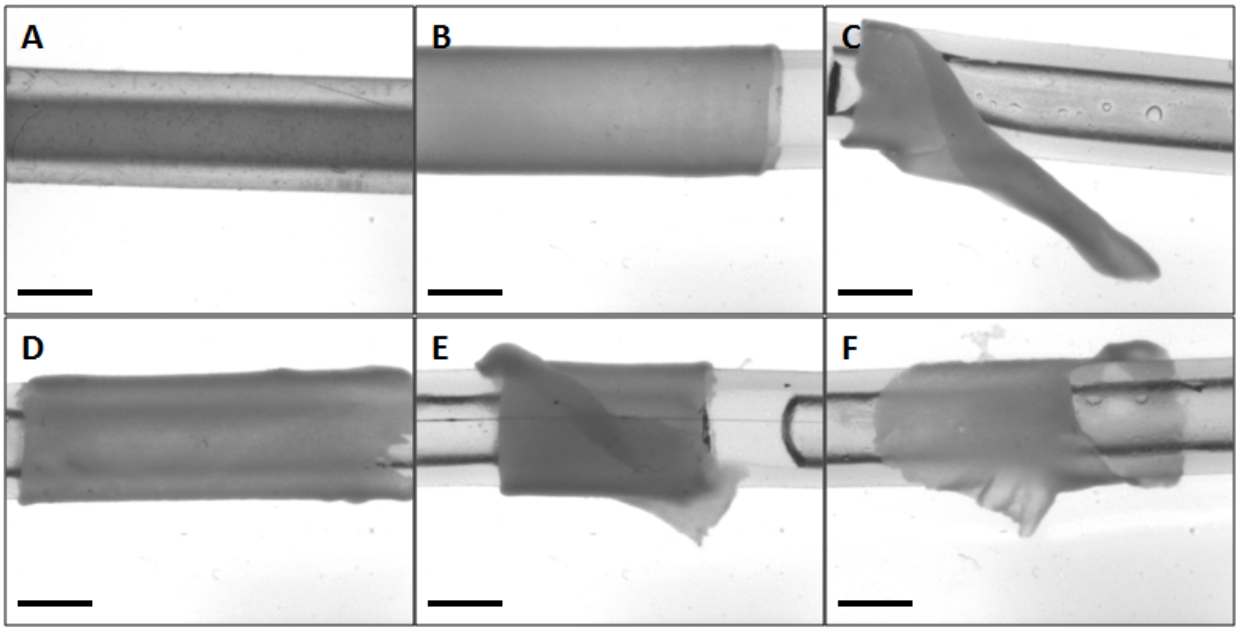

Figure 32 - DVT images of tissue tubes formed during centritubing repeatability analysis. Images were used to measure the tubes' lengths and thicknesses. Control (A) is the silicone tube (central mandrel) alone. Scale bars 2 mm.

It is difficult to make many conclusions from the length data gathered using the DVT images, which was measured to be 8.9 ± 3.5 mm. There is much evidence that tissue tube length is inconsistent, between the quantitative data and the images themselves. Tube 1.021-C appears to be more uniform (Figure 32B), tube 1.026-B appears to have folded over upon itself (Figure 32E), and tube 1.026-D (which could not be measured) contracted circumferentially and folded onto itself (Figure 32C). In terms of wall thickness data, there is also much variability between samples. In general, the wall thickness appears to be thicker for samples with shorter recorded lengths. This suggests that the same number of cells are present, but lateral tissue contraction caused shorter tube length. Unfortunately, there is not enough data in this set to prove this hypothesis.

It is possible that a more adhesive surface for the central mandrel might prevent further contraction, folding, and tearing of the tissue resulting in more consistent tissue tube lengths and thicknesses.

All centritubing observations and results are summarized by experiment and sample number as tables in the Appendix. Overall, tissue tube formation success improved to 85% using a smoother seeding surface, central mandrel, and modified culture apparatus. Tissue tube shape, thickness, and length were not consistent using the current methods. In order to improve the methodology further, several cellular parameters that may impact tube formation were investigated in the next chapter.

Chapter 5 – Cellular Parameters in Centritubing

The aim of the experiments in this chapter was to systematically evaluate primary cellular parameters that could affect tube formation in the centritubing method. Cellular parameters that may play a role in centritubing are cell-surface adhesion, cell-cell and tissue cohesion, spinning force, cell coverage (density), and cell viability (Figure 33).

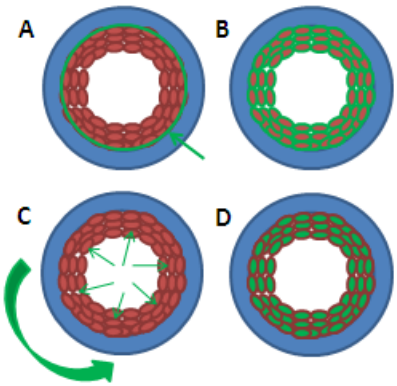

Figure 33 - A schematic representation of cellular factors that affect centritubing success. A) cell-surface adhesion, B) cell-cell and tissue cohesion, C) spinning force, D) cell coverage (density).

Simplified model systems that can provide insight prior to centritubing culture or in 2 dimensions may allow questions to be answered that were confounded in centritubing tube formation. While we have shown an improved rate of success in tissue formation using centritubing, it still requires many cells, precise manufacturing, and meticulous preparation to overcome inconsistencies in tube assembly. These inconsistencies prevent detailed analyses that are needed to optimize the ideal parameters of centritubing, such as cell density, spinning force, spinning time, device materials, culture time, and culture conditions.

In this chapter, our objectives are to:

- Identify the range of adequate cell seeding coverage for centritubing
- Determine if cells are viable after spinning
- Determine if the magnitude of centrifugal force being used is adequate for cell adhesion to a substrate
- Determine if we can improve initial cell aggregation by modifying cell dissociation methods

5.1 – Determining the Range of Cell Densities for Complete Seeding Coverage in Centritubing

The objective of this experiment was to gather information regarding cell density and seeding efficiency in the centritubing method in order to determine if the current seeding density is adequate for complete coverage of the seeding surface and to identify a range of cells that will accomplish complete coverage.

To create a tissue using centritubing, at minimum a complete monolayer of cells (100% confluence) is required. The cells and tissue contract in such a short time period that they must all be attached to one another before this contraction begins around 24-48 hours post-spinning. If the cells are not all attached there will be holes or weak areas in the tissue that cause it to grow in clumps rather than a complete tube. A schematic representation of cell coverage in centritubing is shown in Figure 34. In order to determine a correlation between cell density and confluence, raSMCs were cultured to approximately 85% confluence in a T75 flask (a 75cm^2 surface area). Cells were trypsinized, resuspended, and counted. Using the total cell number and the surface area of the flask it was determined that a cell density of 780 raSMCs/mm^2 is equivalent to 85% confluence. It was then interpolated that approximately 1000 raSMCs/mm^2 would be a confluent monolayer.

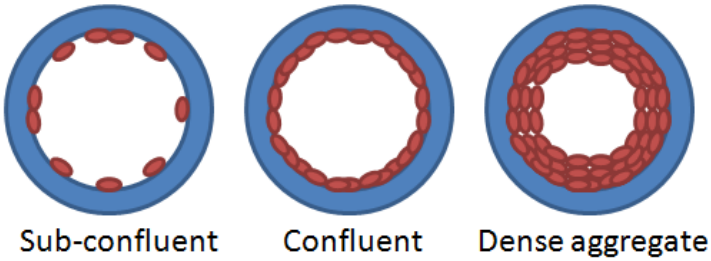

Figure 34 - A schematic representation of confluence. The schematic shows cell coverage on the cylindrical spinning chamber wall. It is clear that at least 100% confluence is necessary to create a tubular construct.

In centritubing, cells were seeded onto a spinning chamber wall that has a surface area of approximately 400 mm^2 (3.175 mm radius, 2 cm deep). In theory, to achieve a confluent layer (1000 raSMCs/mm^2) on this surface only 0.4x10^6 cells are needed. When 20x10^6 cells were centritubed, this resulted in a density of 50,000 raSMCs/mm^2—or about 50 layers of cells.

The purpose of this experiment was to qualitatively observe cell density and test the seeding efficiency of centritubing. From this information, it can be determined which cell amount is ideal for seeding. Seven different concentrations of cell suspensions were created (25x10^6, 16x10^6, 8x10^6, 2x10^6, 0.4x10^6, 0.04x10^6, and the control of 0 cells per tube). These cell suspensions were then each centritubed (n=1 each) for 15 minutes, the media in the spinning chamber was removed for cell counting, and the cells pelletted to the spinning chamber wall were fixed with 70% ethanol for 5 minutes and stained with trypan blue for 5 minutes to visualize cell coverage, then rinsed with PBS. Macroscopic results are displayed in Figure 35.

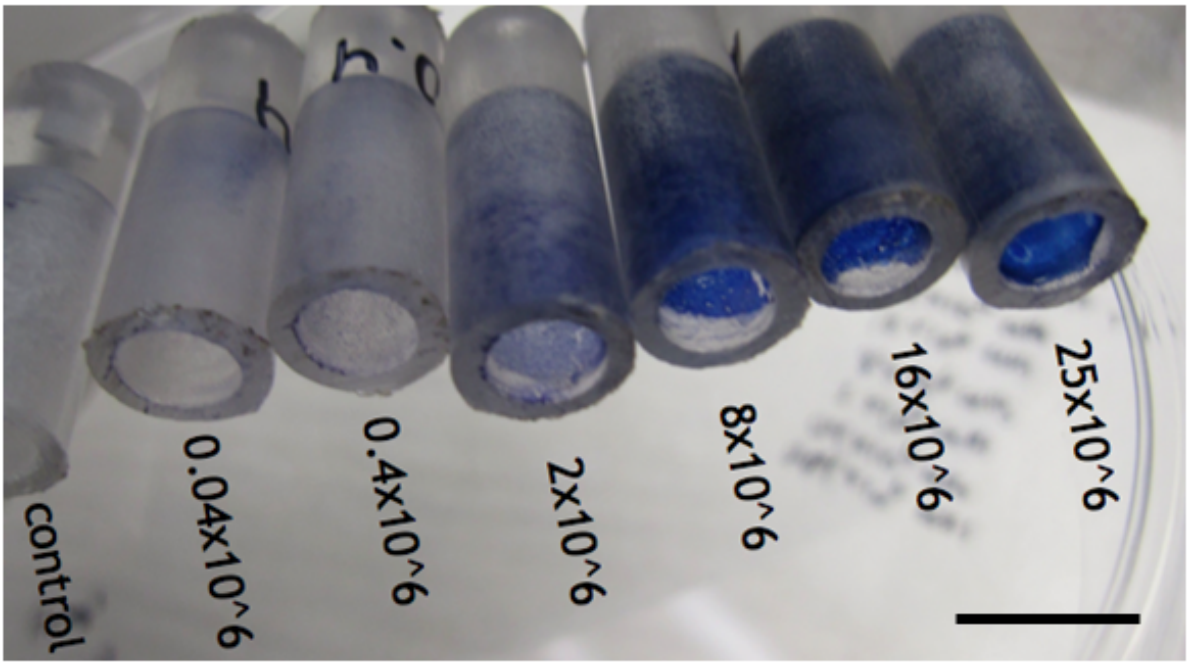

Figure 35 – Cell density within spinning chambers after centritubing. Results suggest that at least $8x10^6$ cells are needed to achieve a complete coverage of cells. Scale bar 1 cm.

There are three distinct groups observed with these results: The 8 $x10^6$, 16 $x10^6$, and $25x10^6$ cell samples showed dense blue coverage, the $2x10^6$ cell sample showed lighter blue coverage, and anything less than $0.4x10^6$ showed minimal blue coverage. We interpret the dense blue as high cell density and several cell layers based on the calculations above. We interpret the lighter blue as lower cell density and a lower incidence of cell-cell contact, but likely still a complete monolayer at the least. Finally, we interpret the minimal blue as an incomplete monolayer.

To determine how many cells were truly seeded onto the spinning chamber, media samples that were removed immediately post-centritubing were suspended to the same volumes and cell counts were performed using a hemocytometer. Results suggested that centritubing has a high seeding efficiency and only a small percentage of cells are not pelletted onto the spinning chamber, as shown in Table 2.

Table 2 – Unattached cells & seeding efficiency in centritubing.

Cells Spun	Cells Unattached Post-Centritubing	Seeding Efficiency
$25x10^6$	$1.62x10^6$	93.5%
$16x10^6$	336,000	97.9%
$8x10^6$	163,500	98.0%
$2x10^6$	124,500	93.8%

5.2 – Viability of Spun Cells vs. Non-spun Cells

The objective of this experiment was to observe if centritubing has a significant negative impact on the viability of raSMCs. Metabolic activity is a common marker for cell viability and replication. An Alamar Blue test was performed to measure the difference in metabolism between cells that were centritubed and cells that were not.

A control standard curve of cells were seeded at 0 cells, 20,000 cells, 40,000 cells, 60,000 cells, 80,000 cells, and 100,000 cells on a 24 well culture plate. Six wells were seeded with 100,000 raSMCs each directly from culture. Six more wells were seeded with 100,000 raSMCs each that were cultured and then delivered through a syringe to simulate injection into the spinning chamber during centritubing. Finally, 30 wells were seeded with 100,000 raSMCs each that were centritubed and then detached from the spinning chamber by pipette and media trituration against the spinning chamber wall. After all wells were seeded 10% (100 µl) Alamar blue was added to each well. Fluorescence readings of 544 nm excitation and 590 nm emission wavelengths were taken at 7 (Figure 36) and 10 hours (Figure 37).

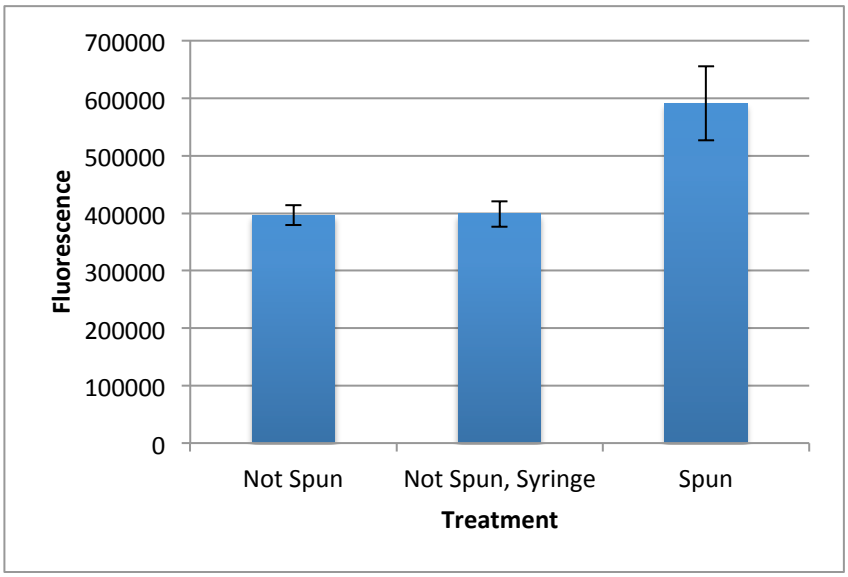

Figure 36 – 7 Hour Alamar Blue Results: Spun vs. Non-Spun Cells at 7 hours. Cells that were spun by centritubing showed significantly more metabolism after 7 hours.

After 7 hours of culture, cells that had been centritubed showed approximately 33% more fluorescence than cells that were not spun and cells that were injected through a syringe needle.

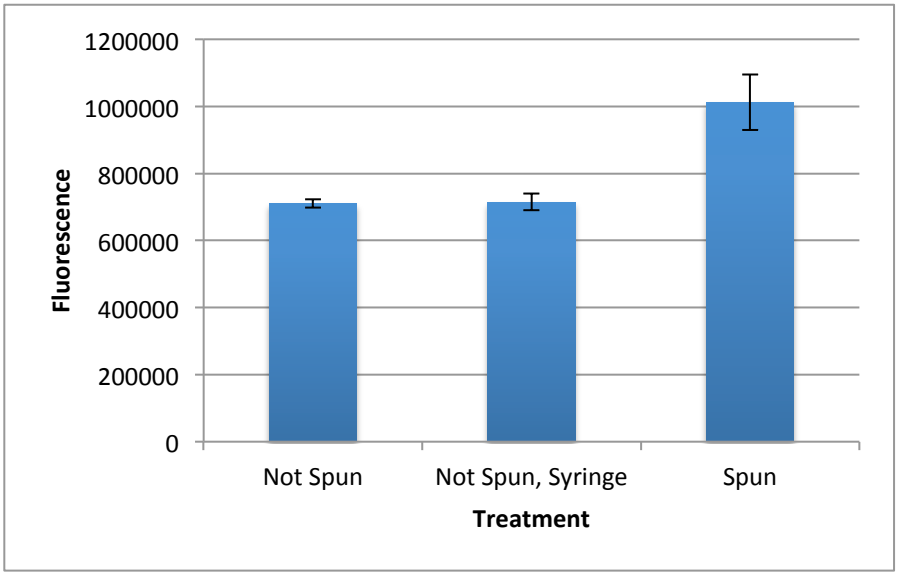

Figure 37 – 10 Hour Alamar Blue Results: Spun vs. Non-Spun Cells at 10 hours.

After 10 hours of culture, cells that had been centritubed continued to show a similar increase in fluorescence over non-spun and syringe-injected cells, possibly due to the increased physical stress being applied to the cells.

5.3 – Effect of Centrifugal Force on Cell Attachment Strength: Proof of Principle

The objective of this study was to understand if there is a relationship between centrifugal force and cell attachment strength. For example, does centrifugation increase cell adhesion strength to a substrate? If this could be understood in a 2-dimensional system, then resources could be utilized more efficiently to determine the optimal amount of force (and therefore spinning speed) for cell attachment. This information could then be translated back to centritubing relating to cell-substrate interaction and centritubing force (Figure 38). This particular study was designed to demonstrate that centrifugation has an impact on cell attachment and is worth investigating in more depth.

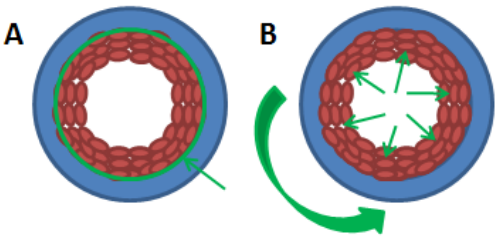

Figure 38 – Schematic of cell adhesion & centrifugal force. This study could provide valuable information regarding cell-substrate interaction (A) and the force acting on cells during spinning (B).

It was hypothesized that centrifugation does increase cell attachment strength over statically seeded cells. This hypothesis was based on the common laboratory procedure of centrifuging cells in

test tubes, which produces a pellet that is more difficult to detach from the test tube and resuspend than cells that have simply settled to the bottom of the test tube due to gravity. To test this, cells were seeded onto plates by centrifugation or by gravity for a short period of time. Then fluid flow was used to disrupt loosely attached and unattached cells for counting. A comparison of the two cell amounts would give an indication as to the strength of cell attachment to the plate for each group.

To do this, raSMCs were first grown to confluence, trypsinized, and resuspended to $1x10^6$ cells/ml. Then, 1 ml of this suspension was added to two wells in two separate 6-well plates (four wells total). Immediately after seeding, these plates were centrifuged at 1000 rpm (179 xg) for 5 minutes. Following centrifugation, the plates were gently shaken (media flowed back and forth twice, then swirled in a circle twice, then back and forth once more) to disturb the cells that may not be attached strongly. The media was collected by pipette and stored in a separate test tube. This procedure was repeated with centrifugation speeds of 2000 rpm, 3000 rpm, and also a static sample that was not centrifuged at all (716 xg, 1610 xg, and 1 xg respectively). Cell counts were then performed on the collected media to determine how many cells were not attached strongly for each group.

Cell counts showed that centrifugation decreased the number of cells that were subject to detachment by gentle fluid flow. A force of 1 xg for 5 minutes was enough for 73% of the seeded cells to attach to the culture surface. Forces of 179, 716, and 1610 xg for 5 minutes were enough to attach 98.4%, 98.4%, and 99.9% of the cells to the culture surface. This suggests that centrifugal force does have a positive impact on initial cell attachment. Data is displayed in Figure 39.

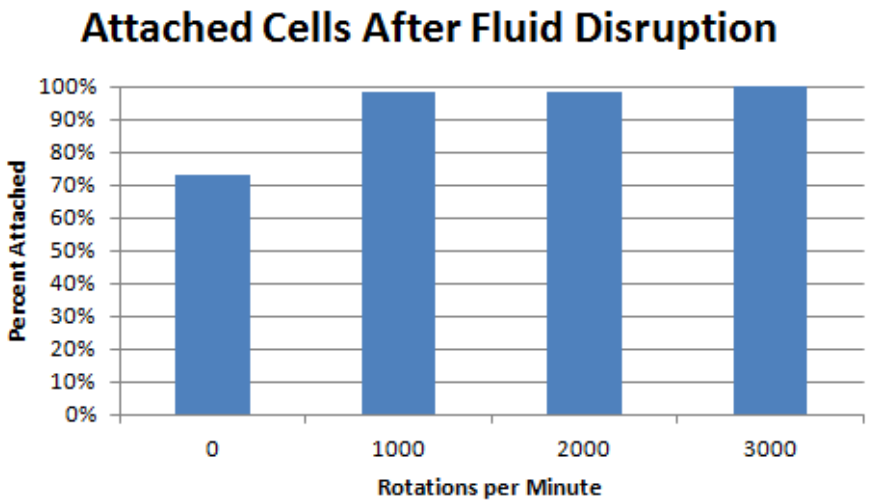

Figure 39 - Cells seeded by centrifugal force resisted detachment flow.

5.4 – Effect of Centrifugal Force on Cell Attachment Strength

After observing the possible improvement in cell attachment strength by centrifugal force, we wanted to perform a more in depth experiment with controlled cell detachment. The goal of this experiment was to determine if the centrifugal force experienced in centritubing is adequate for keeping cells adhered to a surface even with forces acting to detach them. As described in Background section

2.5, centritubing results in a force of about 429.5 xg acting upon the cells for 15 minutes. For this experiment, the force was replicated as close as possible and a force half the magnitude of the attachment force was used to detach the cells over the same time period.

Cells were seeded at a concentration of 100,000 cells/2 ml into three wells of four 12-well plates. Each plate was exposed to a different set of forces for cell attachment and detachment, shown in Table 3. To achieve a seeding force of 355.6 xg, plates were centrifuged at 1440 rpm immediately after the cell suspensions were added to the plate. To achieve a seeding force of 1 xg plates were seeded statically. To achieve a detachment force of 175.3 xg, plates were sealed with parafilm, inverted, and centrifuged at 990 rpm. To achieve a detachment force of 1 xg plates were sealed with parafilm and statically inverted. All samples were unintentionally seeded statically for an extra four minutes prior to the official seeding time recorded (the centrifuge was already in use).

Table 3 - Description of attachment and detachment conditions.

Plate	Seeding Force	Seeding Time	Detachment Force	Detachment Time
Force on – Force off	355.6 xg	15 min	175.3 xg	15 min
Force on – Gravity off	355.6 xg	15 min	1 xg	15 min
Gravity on – Force off	1 xg	15 min	175.3 xg	15 min
Gravity on – Gravity off	1 xg	15 min	1 xg	15 min

After the cells were seeded and subsequently subjected to the respective detachment forces, the media was aspirated, wells were washed with PBS, and attached cells were trypsinized so cell counts could be performed. Results are shown in Figure 40.

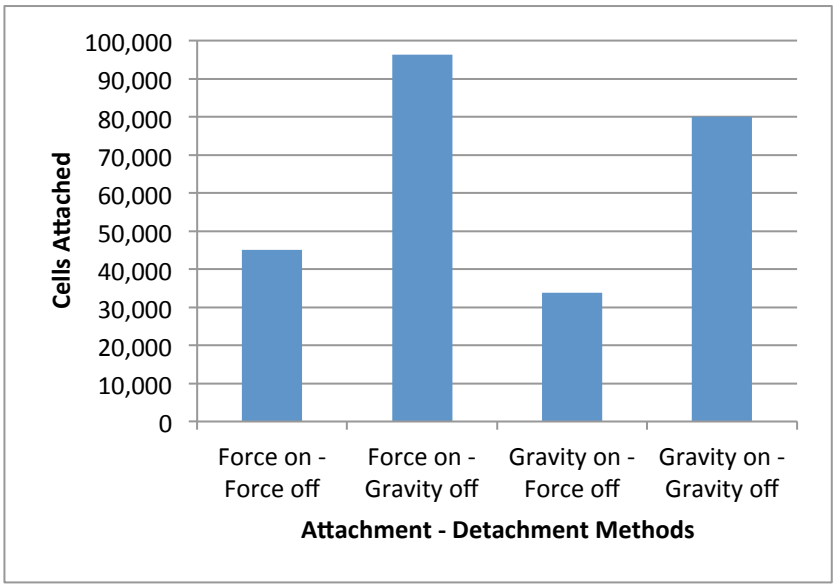

Figure 40 - A graph of attached cells remaining after force applied. Remaining cells (100,000 seeded initially) after the application of detachment forces, suggests that centrifugal force of 355.6 xg is enough to keep cells adhered against the force of gravity for 15 minutes.

In comparing cell counts between plates detached by centrifugal force versus plates detached by gravity, data suggested that using centrifugal force on inverted plates provided more detachment force than gravity. This resulted in fewer cells remaining attached. The Force on – Gravity off sample replicates the seeding and culture of centritubing most accurately, which suggests that the current force used to seed cells is adequate to keep them adhered to the spinning chamber against gravity for 15 minutes. Conversely, the Force on – Force off sample suggests that additional disruptive force will detach cells.

5.5 – The Effects of an Alternative Cell Dissociation Method on Cell Aggregation Over Time

According to the literature (Takeichi, 1977), the calcium chelator EDTA plays a large role in the dissociation of attachment proteins during standard detachment of cells from culture surfaces using trypsin with EDTA. Specifically, EDTA deactivates cadherins, the calcium-dependent bonds between cells. EDTA is normally used because it is less harmful for detaching cells. One group (Duguay, 2002) suggests that using trypsin without EDTA and adding $CaCl_2$ at a 2mM concentration will help preserve these cadherins and will allow cells to aggregate rapidly after detachment.

It would be important to preserve cadherins in centritubing in order to expedite cell aggregation. If cells form faster, stronger bonds as they are pelletted to the surface during spinning, then it may prevent certain disturbances from detaching them and causing a failure in tissue tube formation. This study could provide new methods for improving cell attachment to the spinning chamber and to each other during centritubing (Figure 41).

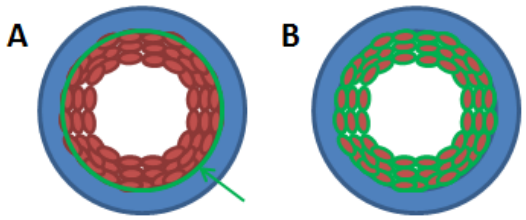

Figure 41 – Schematic of cell adhesion and tissue cohesion. Preserving cadherins may improve cell-spinning chamber adhesion and cell-cell cohesion during centritubing.

To test the differences in cell attachment and aggregation between trypsin with EDTA and with 2mM $CaCL_2$, several experiments were performed. First, raSMCs were detached from three separate 145mm plates using equal amounts (3ml) of trypsin 0.25% with EDTA, trypsin 0.25%, and trypsin 0.25% with 2mM $CaCl_2$ for 5 minutes each. After trypsinization, cells were counted using a hemocytometer and images were taken to compare the detachment between trypsin with EDTA and with $CaCL_2$, specifically to ensure that cells detached with $CaCl_2$ were not remaining aggregated as shown in Figure 42.

Furthermore, live and dead cells were manually counted in trypan blue immediately after trypsinization and resuspension, as shown in Table 4. As expected, counts suggested that viability

decreased slightly when EDTA was not used, but not to the point that EDTA is required to retain a useable amount of viable cells.

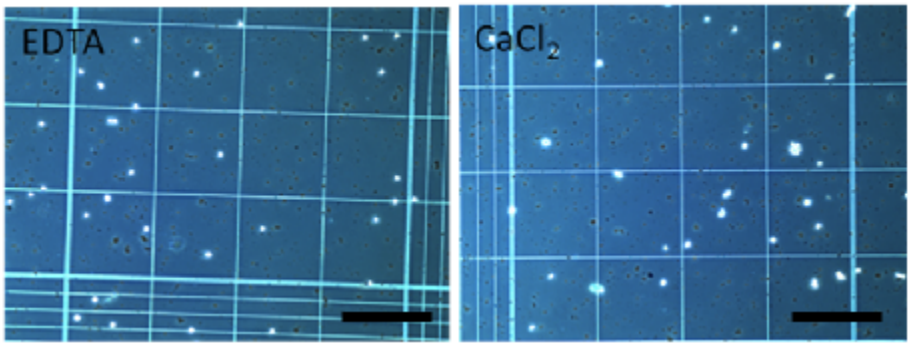

Figure 42 - Images of raSMCs detached by trypsin-EDTA vs trypsin-$CaCl_2$. Results showed that cells detached and did not remain aggregated in either treatment. Scale bars 0.2 mm

Table 4 - Counts of cells detached using different types of trypsinization.

Detachment	Live Count	Dead Count	Live/ml	Dead/ml	Total Cells	Percent Live
Trypsin-EDTA	310	9	775,000	22,500	15.95×10^6	97.2%
Trypsin	243	11	607,500	27,500	12.7×10^6	95.7%
Trypsin-$CaCl_2$	207	12	517,500	30,000	10.95×10^6	94.5%

After cells were counted, each group was resuspended to 600,000 cells/ml. Cells were taken from these suspensions and seeded on two different substrates. Cells detached using trypsin-EDTA, trypsin, and trypsin-$CaCl_2$ were seeded 1 ml per well into 4 wells each on a 12-well tissue culture plate to observe cell attachment to a substrate. The same amounts were seeded into 4 wells each on a 12 well tissue culture plate coated with 2% agarose in DMEM for a non-adhesive surface to observe only cell attachment to other cells.

Images of cells seeded onto tissue culture plates after 18 minutes and 3 hours in culture (Figure 43) suggest that trypsin with $CaCl_2$ allowed cells to aggregate and adhere to one another extremely rapidly. However, it is difficult to make any significant observations regarding cell attachment to the substrate. After three hours each treatment group appeared similar, which suggests that after attachment occurs there is little difference in culture. This is important because in centritubing we are looking for rapid aggregation and strong cell attachment to one another within the first 15-30 minutes (during and immediately after spinning), which results in normal cell behavior long-term.

Images of cells seeded onto agarose-coated tissue culture plates after 25 minutes and 5 hours support the observations on the tissue culture plate samples (Figure 44). Within 25 minutes after seeding, trypsin and trypsin-EDTA samples showed individual cells floating in the wells, while the trypsin-$CaCl_2$ sample already showed cell clumps beginning to form. Similarly, after 5 hours, the trypsin and trypsin- $CaCl_2$ samples shows large floating sheets of cells, while the trypsin-EDTA samples still

contained almost exclusively individual cells floating. This could be seen even more readily when the media in the samples was swirled and cell-clump motion was observed under light microscopy.

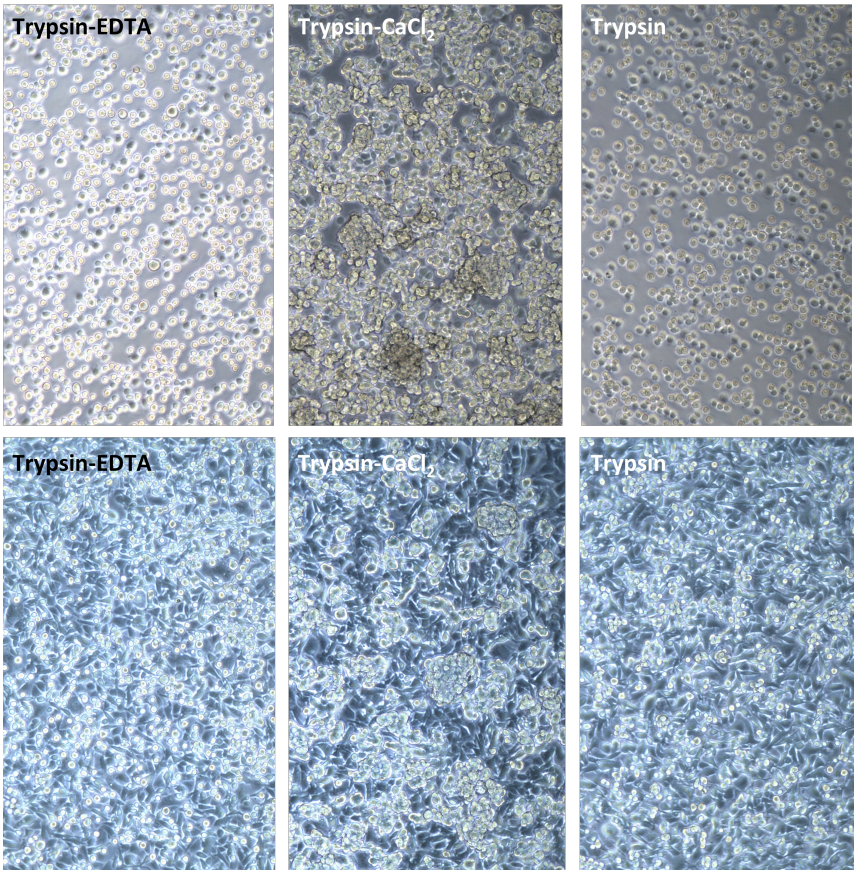

Figure 43 – Dissociated cells seeded on culture plastic at 18 minutes. TOP ROW: 18 minutes after seeding on tissue culture plastic only the trypsin-$CaCl_2$ sample showed signs of cell aggregation, but the trypsin only sample showed signs of cell attachment and spreading. BOTTOM ROW: After 3 hours, all wells were overconfluent and cells were attached similarly, although there were still more aggregated cell clumps in the trypsin-$CaCl_2$ sample.

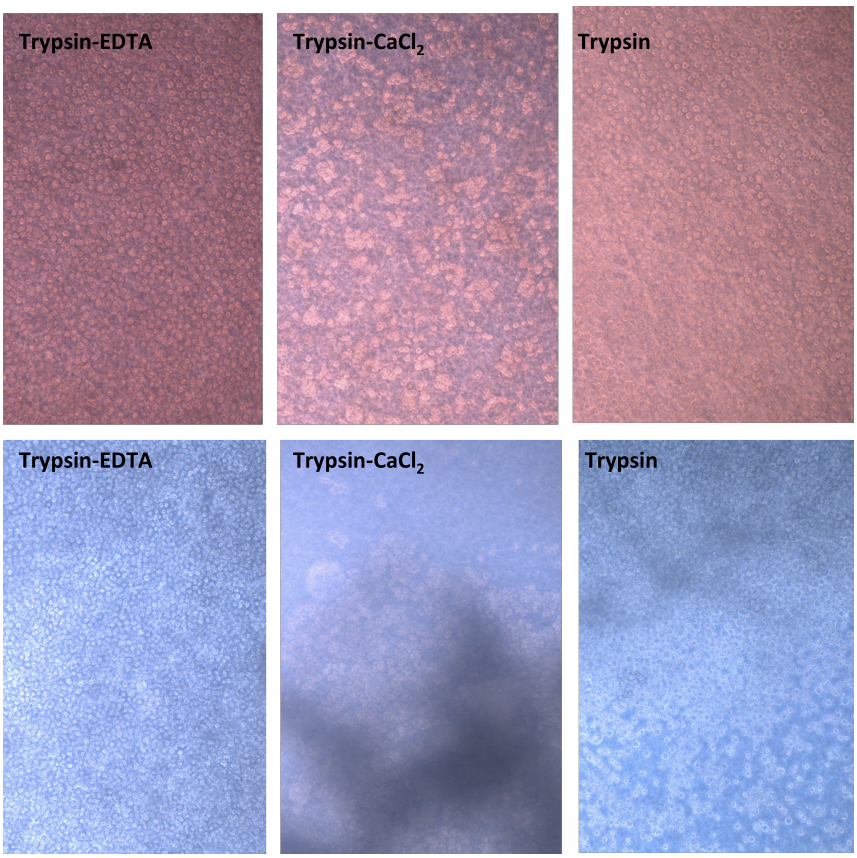

Figure 44 - Dissociated cells seeded on agarose at 25 minutes. TOP ROW: 25 minutes after seeding on agarose only the trypsin-$CaCl_2$ sample showed signs of cell aggregation, while the other samples had only individual cells floating. BOTTOM ROW: After 5 hours, there were larger cell clumps in the trypsin-$CaCl_2$ sample, the trypsin only sample began to have some cell aggregation, and the trypins-EDTA sample remained almost exclusively individual cells.

Chapter 6 – Discussion & Conclusions

In the present study, we have investigated the feasibility of rapidly aggregating cells into an entirely cell-derived, small diameter tubular construct by implementing modifications to centritubing. By adding a central mandrel, manufacturing a smoother seeding surface, and developing an improved culture apparatus, we were able to create cellular tube constructs with a lumen diameter of 3.2 mm at a construct formation success rate of 85%. The results of this study indicate that centritubing can be a successful method for tubular tissue formation, but that several adjustments in methodology are still needed to improve consistency in construct formation success and construct dimensions.

The three primary modifications to our previous work with centritubing were the central mandrel, the smoother seeding surface, and the culture apparatus. During this project, an improved culture apparatus that was sterile and did not leak improved tube formation success from 0% (0/11) to 43% (3/7). In experiments using the improved culture apparatus, the modification to spinning chamber manufacturing that resulted in a smoother seeding surface doubled tube formation success from 43% (3/7) to 85% (11/13), as shown in Figure 45. Finally, the central mandrel successfully acted as a substrate for tissue contraction for all samples, and sustained tube formation in 70% of the total samples centritubed.

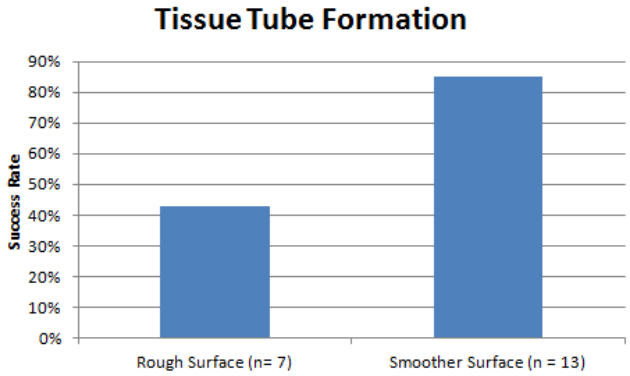

Figure 45 – Tissue tube success after design modifications.
After incorporating the central mandrel and modified culture apparatus, tube formation success increased from 43% to 85% with the manufacture of a smoother seeding surface.

The results of this study suggest that cellular tube constructs with a lumen diameter of 3.2 mm will form rapidly using a specific set of parameters:

- A non-adhesive central mandrel 3.2 mm in diameter
- A "smoother" cell seeding surface
- Vertical culture in a sterile and non-leaking culture apparatus
- A secure motor-spinning chamber interface for a smooth, uniform spin
- Minimal disruption of the spinning chamber after spinning
- $20x10^6$ raSMCs
- 15 minute spin
- 11,000 rpm (429.5 xg)
- 3 days of culture in growth media

The end product of centritubing under the aforementioned conditions is a cellular tube construct contracted around the central mandrel with a clinically relevant lumen diameter of 3.2 mm, wall thickness ranging between 300 – 450 μm, and a length between 5 – 15 mm. The measured wall thickness of these constructs is greater than the 200 μm nutrient diffusion limitation through tissue (Muschler, 2004), however Niklason, et.al. have created TEBVs with 200 – 400 μm wall thicknesses and shown native vessel thickness to be 300 μm (Niklason, 1999). These values are similar, but TEBVs created by Niklason, et.al. are a late stage product cultured 5 – 8 weeks under pulsatile and non-pulsatile luminal flow, while our cellular tube constructs are only the initial step in creating a TEBV using centritubing. Additional downstream conditioning will be needed to culture these centritubed tissue tubes into clinically relevant TEBVs.

In order to achieve the 3.2 mm diameter cellular tube constructs as the first step in centritubing, it is likely that tissue thickness is less important than the number of cell layers in the construct. Based on our calculations in Results section 5.1, centritubing with $20x10^6$ cells theoretically results in a construct that is 50 cell layers thick on a flat surface. Assuming an individual cell is 10 μm thick, the maximum number of cell layers seeded should be 20 in order to stay within the 200 μm diffusion limit. These calculations suggest that a density of 20,000 cells/mm^2 ($8x10^6$ cells seeded in centritubing) is the maximum limit. However, because the spinning chamber surface is not flat, it is likely that the true limit for centritubing is slightly higher.

L'Heureux, et.al. have shown the ability to create completely cell-derived TEBVs using fibroblasts, which have a burst pressure strength of 3490 ± 892 mmHg (compared to 3196 ± 1264 mmHg for the internal mammary artery used for CABG) (Konig, 2009). It is difficult to compare the strength of our 3 day cellular tube constructs to these TEBVs that have been cultured 6-8 weeks as sheets in ascorbic acid supplemented media to improve collagen production and then further cultured for 12 weeks as tubes. A more appropriate comparison in strength might be found in rapidly self-assembled cellular aggregates, such as spheroids and rings. We did not measure centritubed tissue strength in our studies to date.

Centritubing creates un-conditioned cellular aggregates that are manipulated into a tubular formation. The cellular tube constructs created by centritubing are likely to be closely related to other self-assembled aggregates, such as spheroids and toroids (rings). Napolitano, et.al. created a method for rapid self-assembly of cells into spheroids and toroids, which exhibit circumferential contraction immediately after aggregation—similar to tubes in centritubing (Napolitano, 2007). They were able to quantify the power of toroid contraction to be 4.3 ± 1.7 pJ/h for fibroblast cells using the distance travelled up a sloped peg (Youssef, 2011). We believe similar contraction strength and mechanics are applied when aggregated SMCs contract off the spinning chamber wall in centritubing. Our lab also developed a method to create 2-6 mm diameter raSMC tissue ring aggregates, which have an ultimate tensile strength up to 503 ± 76 kPa cultured for 8 days in growth medium (Gwyther, 2011). Again, these values are low compared to the strength necessary to replace native arteries for clinical applications (for example, the porcine carotid artery has a UTS approximately 6.6 MPa) (Dahl, 2007). These low values could be due to the lack of a mature ECM (Gwyther, 2011).

Although we were able to improve the consistency of success in creating centritubed cellular constructs, there were still several complications relating to device design and handling that resulted in tube failure. Future design considerations should avoid complications that we experienced, including:

- Bacterial contamination due to one-time use components
- Incomplete injection of cell suspension
- Wobbling of spinning chamber during spin
- Excessive motor vibration during spin
- Silicone cap detachment
- Cap leaking during spin
- Rough inner surface in the cylindrical seeding chambers
- Silicone cap-central mandrel adhesion
- Disruption of seeded cells prior to aggregation due to spinning chamber handling or seeding surface disturbance
- Culture apparatus leaking
- Difficulty harvesting tubes from the spinning chamber after culture
- Uncontrolled tissue contraction

These complications often occurred sporadically and were difficult to avoid entirely under the current device design and methods. In several instances it was impossible to tell if these complications truly affected tube formation, resulting in confounding observations for causes of tube failure. Testing spinning and cellular parameters (such as spin speed, cell density, spin time, media formulation, etc.) in two-dimensions may provide a simpler, more controlled means of analyzing centritubing conditions in future studies, as suggested by the results in Chapter 5.

Chapter 7 – Future Work

This section discusses future work that may support the improvements made during the present study, further optimize the centritubing process, and investigate the next steps that lead to mature tubular construct culture.

7.1 – Improving Cellular Tube Construct Formation

While we have shown that centritubing is a process that can effectively aggregate a cell suspension into a cellular tube construct contracted around a non-adhesive mandrel, there are still some design changes that could improve the success rate of tube formation and limit many of the complications that we observed throughout this project. We would like to investigate several interactions that we believe to be integral to cellular tube formation in centritubing, including cell-spinning chamber adhesion, cell aggregate cohesion, and tissue contraction onto the central mandrel. We believe that these three interactions are significantly interrelated, in that each one may have an effect on the others. When the aggregated cells begin to contract during culture, the contraction forces are acting in two ways: 1) They are pulling cell away from one another and their ECM, and 2) they are pulling the tissue aggregate away from the spinning chamber surface. The latter of these forces is desirable, while the former is not. In order to ensure that the tissue aggregate contracts off the spinning chamber surface, but does not contract so much as to rip itself apart, we hypothesize that the cell-surface adhesion must be weaker than the cell-cell and cell-ECM attachment within the tissue.

To quantify the cell-cell and cell-ECM cohesion within the tissue aggregate, cell-derived ring models could be used. These tissue rings have been extensively studied in our lab (Gwyther, 2011) and can be created using the same lumen diameter, cell type, and media formulation as in centritubing. We began to see tissue contraction in the spinning chambers by 2 days, so these rings could be harvested and used for uniaxial tensile testing to determine the ultimate tensile stress, which would be directly related to the strength of the cell-cell and cell-ECM bonds within the tissue. Understanding the strength of the cohesion and adhesion of tissue in centritubing may help to prevent tissue tearing during contraction.

We began investigating the strength of the cell-surface interactions by performing some inverted centrifugation assays on tissue culture plastic (Results section 5.3-5.4), however, these results are conceptual and do not translate directly to the materials used in centritubing. Polycarbonate sheet molds developed in our lab (Appendix section A.2) may be used to achieve a more accurate quantification of the cellular adhesion to polycarbonate after applying centrifugal force (429.5 xg as in centritubing) for 15 minutes.

Understanding the cell-spinning chamber adhesion, cell aggregate cohesion, and tissue contraction is valuable for optimizing centritubing, however, we must also continue to make modifications for the manufacture and preparation of the centritubing device in order to increase the consistency of tube formation by centritubing.

7.2 – Optimizing the Centritubing Device and Process

Throughout the present study, we observed several complications that may have resulted in tube formation, including but not limited to a rough seeding surface, silicone cap-central mandrel adhesion, and leaking/incomplete filling of the spinning chamber before centritubing.

While we were able to create a smoother seeding surface within the spinning chamber by modifying our manufacturing method slightly, this has not been quantified. We believe that a surface that has a minimal number of imperfections and is as smooth as available manufacturing methods allow would provide a more consistent substrate for cell attachment and tissue growth. In future work, we would like to modify our manufacturing methods further to improve the smoothness of the seeding surface.

Our lab has already investigated possible modifications to prevent silicone cap-central mandrel adhesion and leaking/incomplete filling of the spinning chamber. This new cap system has not been tested, however, we believe that it is a step in the right direction (Figure 46 D, E). Ideally, a cap should incorporate the central mandrel and allow for media and nutrient exchange to avoid media contact with the outside of the spinning chamber (a contamination risk). Potentially, two separate caps could be designed—one for during spinning and one for during culture.

Our lab has also begun work to streamline the centritubing process. We have designed a centritubing system that will spin the cells in a spinning chamber for 15 minutes automatically at the press of a button (Figure 46 A, B, C). However, this system has not been tested using cells and was measured to spin at fewer rotations per minute than we have used in centritubing (Figure 47). This system should be tested for cell adhesion and coverage using trypan blue for spatial observations (similar to Results section 5.1).

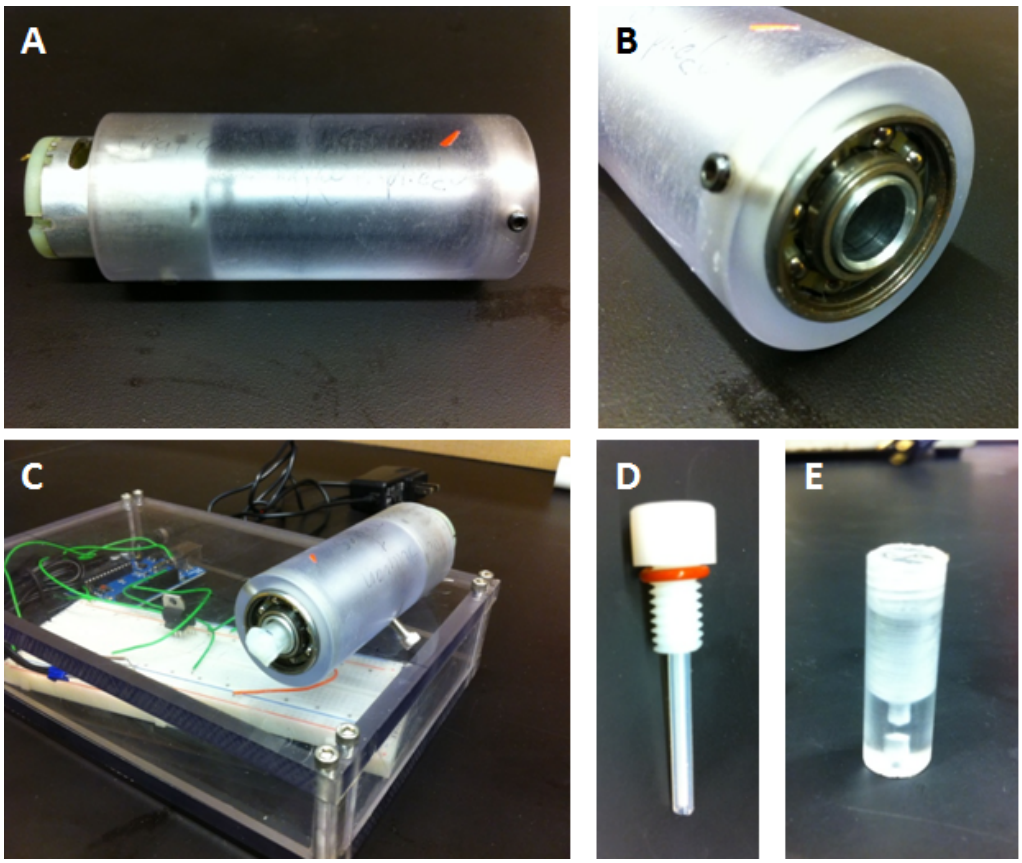

Figure 46 - The modified centritubing device.
The device was comprised of A) a motor and spinning chamber holder, B) a ball bearing to allow spinning within the holder, C), a circuit to control spinning time, D) a modified screw-on cap with attached central mandrel, and E) a modified spinning chamber to complement the screw-on cap.

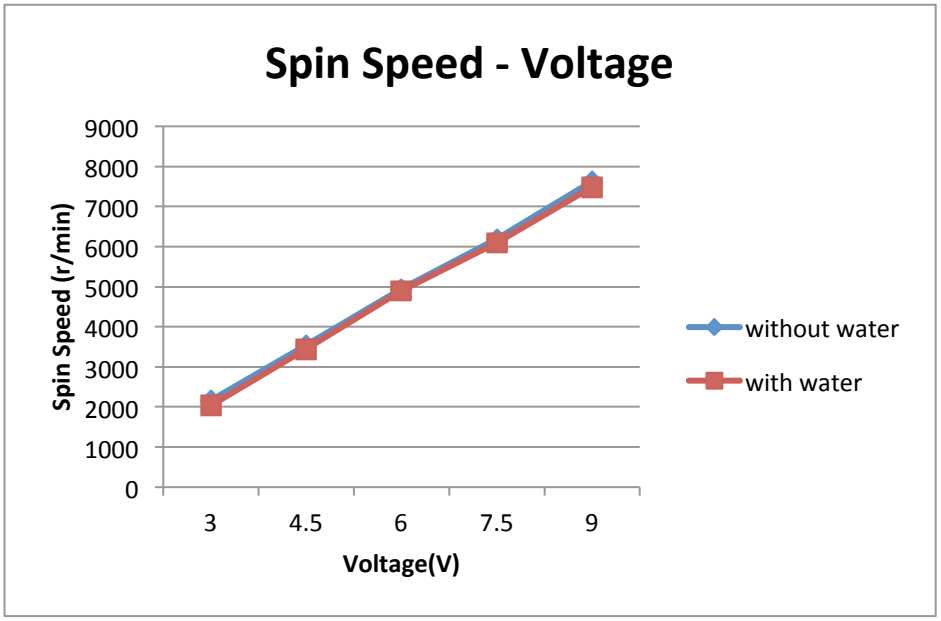

Figure 47 – Spin speed of modified centritubing device.
A laser tachometer was used to measure the rotations per minute of a spinning chamber spun with and without water using the modified device. (n = 3)

7.3 – The Next Steps

Centritubing is still in the early stages of development. The above recommendations should improve the simplicity and overall consistency in tube formation and success. Once the process of using centritubing to achieve a contracted cellular tube construct has been optimized, there is still much downstream work that will be required to culture it into a final TEBV product. The primary element missing from cellular tube constructs created from centritubing is an organized, functional matrix.

While native blood vessels synthesize a matrix comprised of collagen and elastin, it has been shown that collagen production alone provides enough strength to achieve clinically acceptable burst pressures in TEBVs (McAllister, 2009). Our lab and others have shown that media supplements such as ascorbic acid and amino-caproic acid can increase collagen synthesis *in vitro* (L'Heureux, 1998; Adebayo, 2012). Furthermore, mechanical stimulation, such as cyclic strain has been shown to increase collagen production and organize collagen fibers (Niklason, 1996; Solan, 2009; Gauvin, 2011; Syedain, 2011). Long-term culture under these conditions could enable centritubed cellular tube constructs to mature into mechanically robust TEBVs.

References

Absolom DR, Neumann AW, Zingg W, van Oss CJ. Thermodynamic studies of cellular adhesion. *Trans Am Soc Artif Intern Organs*. 1979. 25: 152-158

Absolom DR, Thomson C, Hawthorn LA, Zingg W, Neumann AW. Kinetics of cell adhesion to polymer surfaces. *J Biomed Mater Res*. 1988. 22(3): 215-229

Adebayo O, Hookway T, Hu JZ, Billiar KL, Rolle MW. Self-assembled smooth muscle cell tissue rings exhiit greater tensile strength than cell-seeded fibrin or collagen gel rings. *J Biomed Mater Res A*. 2012

Ahlfors JW, Billiar KL. Biomechanical and biochemical characteristics of a human fibroblast-produced and remodeled matrix. *Biomaterials*. 2007. 28: 2183-2191

Ahmann KA, Weinbaum JS, Johnson SL, Tranquillo RT. Fibrin degradation enhances vascular smooth muscle cell proliferation and matrix deposition in fibrin-based tissue constructs fabricated *in vitro*. *Tissue Engineering*. 2010. 16(10): 3261-3270

Aikawa M, Sakomura Y, Ueda M, Kimura K, Manabe I, Ishiwata S, Komiyama N, Yamaguchi H, Yazaki Y, Nagai R. Redifferentiation of smooth muscle cells after coronary angioplasty determined via myosin heavy chain expression. *Circulation*. 1997. 96: 82-90

Ainslie KM, Garanich JS, Dull RO, Tarbell JM. Vascular smooth muscle cell glycocalyx influences shear stress-mediated contractile response. *J Appl Physiol*. 2005. 98: 242-249

Akamine Y, Kakudo K, Kondo M, Ota K, Muroi Y, Yoshikawa H, Nakata K. Prolonged matrix metalloproteinase-3 high expression after cyclic compressive load on human synovial cells in three-dimensional cultured tissue. *Int J Oral Maxillofac Surg*. 2012. 41: 874-881

Bao B, Jiang J, Yanase T, Nishi Y, Morgan JR. Connexon-mediated cell adhesion drives microtissue self-assembly. *FASEB J*. 2011. 25 (1): 255-264

Bell E. Tissue Engineering: A Perspective. *Journal of Cellular Biochemistry*. 1991. 45: 239-241

Berthold Hermle GmbH & Co. Instruction Manual for Laboratory Centrifuge.

Billiar KL, Throm AM, Frey MT. Biaxial failure properties of planar living tissue equivalents. *J Biome Mater Res A*. 2005. 73A(2): 182-191

Boettiger D, Wehrle-Haller B. Integrin and glycocalyx mediated contributions to cell adhesion identified by single cell force spectroscopy. *J Phys: Condens Metter*. 2010. 22

Boettiger D. Mechanical control of integrin-mediated adhesion and signaling. *Current Opinion in Cell Biology*. 2012. 24

Burford J, Jones C, Soderbom L, Sullivan P. A method for the tubular self-assembly of cells to create tissue engineered blood vessels. 2010. WPI BS Major Qualifying Project. E-project-042210-182526: <http://www.wpi.edu/Pubs/E-project/Available/E-project-042210-182526/>

Cohn JD, Korver KF. Selection of saphenous vein conduit in varicose vein disease. *Ann Thorac Surg*. 2006. 81: 1269-1274

Cole JH, Jones EL, Craver JM, Guyton RA, Morris DC, Douglas JS, Ghazzal Z, Weintraub WS. Outcomes of repeat revascularization in diabetic patients with prior coronary surgery. *Journal of the American College of Cardiology*. 2002. 40(11): 1968-1975

Dahl SLM, Vaughn ME, Niklason LE. An ultrastructural analysis of collagen in tissue engineered arteries. *Ann Biomed Eng*. 2007. 35(10): 1749-1755

Dangas G, Kuepper F. Restenosis: Repeat Narrowing of a Coronary Artery: Prevention and Treatment. *Circulation.* 2002. 105: 2586-2587.

Dean DM, Morgan JR. Cytoskeletal-mediated tension modulates the directed self-assembly of microtissues. *Tissue Engineering A*. 2008. 14(12): 1989-1997

Dinh TL, Veves A. The efficacy of Apligraf in the treatment of diabetic foot ulcers. *Plastic Reconstructive Surgery*. 2006. 117(Suppl.): 152S

Doshi KJ. Direct cell seeding on collagen-coated silicone mandrels to generate cell-derived tissue tubes. WPI MS Thesis. 2009. etd-082809-152033: <http://www.wpi.edu/Pubs/ETD/Available/etd-082809-152033/>

Du J, Che P, Wang Z, Aich U, Yarema KJ. Designing a binding interface for control of cancer cell adhesion via 3D topography and metabolic oligosaccharide engineering. *Biomaterials*. 2011. 32: 5427-5437

Duguay D, Foty RA, Steinberg MS. Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. *Developmental Biology*. 2003. 253: 309-323

Ehrenreich M, Ruszczak Z. Update on tissue-engineered biological dressings. *Tissue Engineering*. 2006. 12(9): 2407-2424

Eierman DF, Johnson CE, Haskill JS. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. *J Immunol*. 9189. 142(6): 1970-1976

Ekstrand J, Razuvaev A, Folkersen L, Roy J, Hedin U. Tissue factor pathway inhibitor-2 is induced by fluid sheat stress in vascular smooth muscle cells and affects cell proliferation and survival. *Journal of Vascular Surgery*. 2010. 52(1): 167-175

Freed LE, Guilak F. Chapter Eleven: Engineering Functional Tissues. In R Lanza, R Langer, & J Vacanti, *Principles of Tissue Engineering.* (3rd ed.) 2007. (pp. 138) Elsevier Inc.

Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension*. 1994. 23(6 Pt 2): 1121-1131

Garcia AJ, Ducheyne P, Boettiger D. Quantification of cell adhesion using a spinning disc device and application to surface-reactive materials. *Biomaterials*. 1997. 18(16): 1091-1098

Gauvin R, Parenteau-Bareil R, Larouche D, Marcoux H, Bisson F, Bonnet A, Auger FA, Bolduc S, Germain L. Dynamic mechanical stimulations induce anisotropy and improve the tensile properties of engineered tissues produces without exogenous scaffolding. *Acta Biomaterialia*. 2011. 7(9): 3294-3301

Ghanaati S, Unger RE, Webber MJ, Barbeck M, Orth C, Kirkpatrick JA, Booms P, Motta A, Migliaresi C, Sader RA, Kirkpatrick. Scaffold vascularization *in vivo* driven by primary human osteoblasts in concert with host inflammatory cells. *Biomaterials*. 2011. 32: 8150-8160

Gong Z, Niklason LE. Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs). *FASEB J*. 2008. 22(6): 1635-1648

Gwyther TA, Hu JZ, Christakis AG, Skorinko JK, Shaw SM, Billiar KL, Rolle MW. Engineered vascular tissue fabricated from aggregated smooth muscle cells. *Cells Tissues Organs*. 2011. 194: 13-24

Gwyther TA, Hu JZ, Billiar KL, Rolle MW. Directed cellular self-assembly to fabricate cell-derived tissue rings for biomechanical analysis and tissue engineering. *J Vis Exp*. 2011. 57: e3366

Hibino N, Shin'oka T, Matsumura G, Ikada Y, Kurosawa H. The tissue-engineered vascular graft using bone marrow without culture. *J Thorac Cardiovasc Surg*. 2005. 129: 1064-1070

Hibino N, McGillicuddy E, Matsumura G, Ichihara Y, Naito Y, Breuer C, Shinoka T. Late-term results of tissue engineered vascular grafts in humans. *J Thorac Cardiovasc Surg*. 2010. 139: 431-436

Hertl W, Ramsey WS, Nowlan ED. Assessment of cell-substrate adhesion by a centrifugal method. *In Vitro*. 1984. 20(10): 796-801

Hirai J, Matsuda T. Venous reconstruction using hybrid vascular tissue composed of vascular cells and collagen: tissue regeneration process. *Cell Transplant*. 1996. 5(1): 93-105

Hu JZ, Billiar KL, Pins GD, Rolle MW. Assessment of ascorbic acid effects on the properties of cell-derived tissue rings. WPI MS Thesis. 2010

Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. *Cric Res*. 2006. 98: 25-35

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Experimental Cell Research*. 1998. 238: 265-272

Kang H, Fan Y, Deng X. Vascular smooth muscle cell glycocalyx modulates shear-induced proliferation, migration, and NO production responses. *Am J Physiol Heart Circ Physiol*. 2011. 300: H76-H83

Kang KL, Lee SW, Ahn YS, Kim SH, Kang YG. Bioinformatic analysis of responsive genes in two-dimension and three-dimension cultured human periodontal ligament cells subjected to compressive stress. *J Periodontal Res*. ePub 23 Jul 2012.

Khan OF, Chamberlain MD, Sefton MV. Toward an in vitro vasculature: differentiation of mesenchymal stromal cells within an endothelial cell-seeded modular construct in a microfluidic flow chamber. *Tissue Engineering A*. 2011. 18(7-8): 744-756

Kim DH, Heo SJ, Kim SH, Shin JW, Park SH, Shin JW. Shear stress magnitude is critical in regulating the differentiation of mesenchymal stem cells even with endothelial growth medium. *Biotechnol Lett*. 2011. 33(12): 2351-2359

Kobayashi N, Yasu T, Ueba H, Sata M, Hashimoto S, Kuroki M, Saiot M, Kawakami M. Mechanical stress promotes the expression of smooth muscle-like properties in marrow stromal cells. *Experimental Hematology*. 2004. 32: 1238-1245

Kohsaka S, Goto M, Virano S, Lee V, Aoki N, Elayda MA, Reul RM, Wilson JM. Long-term clinical outcome of coronary artery stenting or coronary artery bypass grafting in patients with multiple-vessel disease. *J Thorac Cardiovasc Sug*. 2008. 136: 500-506

Konig G, McAllister TN, Dusserre N, Garrido SA, Iyican C, Marini A, Fiorillo A, Avila H, Wystrychowski W, Zagalski K, Maruszewski M, Jones AL, Cierpka L, del la Fuente L, L'Heureux N. Mechanical properties of completely autologous tissue engineered blood vessels compared to human saphenous vein and mammary artery. *Biomaterials*. 2009. 30:1542-1550

Laschke MW, Strohe A, Scheuer C, Eglin D, Verrier S, Alini M, Pohlemann T, Menger MD. In vivo biocompatibility and vascularization of biodegradable porous polyurethane scaffolds for tissue engineering. *Acta Biomaterialia*. 2009. 5: 1991-2001

Lee J, Cuddihy MJ, Kotov NA. Three-dimensional cell culture matrices: state of the art. *Tissue Engineering B*. 2008. 14(1): 61-86

Lemire JM, Covin CW, White S, Giachelli CM, Scwartz SM. Characterization of cloned aortic smooth muscle cells from young rats. *Am J Pathol.* 1994. 144(5): 1068-1081

Lemire JM, Potter-Perigo S, Hall KL, Wight TN, Schwartz SM. Distinct rat aortic smooth muscle cells differ in versican/PG-M expression. *Arterioscler Thromb Vasc Biol.* 1996. 16(6): 821-829

L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. *FASEB*. 1998. 12: 47-56

L'Heureux, N, Dusserre N, Konig G, Victor B, Keire P, Wight T, Chronos N, Kyles A, Gregory C, Hoyt G, Robbins R, McAllister T. Human Tissue Engineered Blood Vessel For Adult Arterial Revascularization. *Nat Med*. 1998. 12(3): 361–365

Li D, Zhou J, Chowdhury F, Cheng J, Wang N, Wang F. Role of mechanical factors in fate decisions of stem cells. *Regen Med*. 2011. 6(2): 229-240

Lysaght MJ, Jaklenec A, & Deweerd E. Great expectations: private sector activity in tissue engineering, regenerative medicine, and stem cell therapeutics. *Tissue Engineering Part A.* 2008. 14 (2): 305-15

McAllister TN, Maruszewski M, Garrido SA, Wystrychowski W, Dusserre N, Marini A, Zagalski K, Fiorillo A, Avila H, Manglano X, Antonelli J, Kocher A, Zembala M, Cierpka L, de la Fuente LM, L'Heureux N. Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study. *Lancet*. 2009. 373: 1440-1446

McClay DR, Hertzler PL. Quantitative measurement of cell adhesion using centrifugal force. *Curr Protoc Cell Bio*. 2001 May. Chapter 9: Unit 9.2

Mitchell, SL, & Niklason, LE. Requirements for growing tissue-engineered vascular grafts. *Cardiovascular Pathology*. 2003. 12(2): 59-64

Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. *J Bone Joint Surg Am*. 2004. 86-A

Napolitano AP, Chai P, Dean DM, Morgan JR. Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Engineering*. 2007. 13(8): 2087-2094

Nasseri B, Pomerantseva I, Kaazempur-Mofrad M, Sutherland F, Perry T, Ochoa E, et al. Dynamic rotational seeding and cell culture system for vascular tube formation. *Tissue Engineering*. 2003. 9(2): 291-299

Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R. Functional arteries grown in vitro. *Science*. 1999. 284(5413): 489-93.

Nilsson J, Ksiazek T, Thyberg J, Wasteson A. Cell surface components and growth regulation in cultivated arterial smooth muscle cells. *J Cell Sci*. 1983. 64: 107-121

Norotte C, Marga FS, Niklason LE, Forgas G. Scaffold-free tissue engineering using bioprinting. *Biomaterials*. 2009. 30(30): 5910-5917

Pankajakshan D, Krishnan V K, Krishnan LK. Vascular tissue generation in response to signaling molecules integrated with a novel poly(e-caprolactone)-fibrin hybrid scaffold. *J Tissue Eng Regen Med*. 2007. 1: 389-397

Pasic M, Muller-Glauser W, Von Segesser L, Lachat M, Turina M, Odermatt B. Endothelial cell seeding improves patency of synthetic vascular grafts: Manual versus automatized method. *European Journal of Cardio-Thoracic Surgery*. 1996. 10(5): 372-379

Pelaez D, Arita N, Cheung HS. Extracellular signal-regulated kinase (ERK) dictates osteogenic and/or chondrogenic lineage commitment of mesenchymal stem cells under dynamic compression. *Biochemical and Biophysical Research Communications*. 2012. 417: 1286-1291

Peyton SR, Kim PD, Ghajar CM, Seliktar D, Putnam AJ. The effects of matrix stiffness and RhoA on the phenotypic plasticity of smooth muscle cells in a 3-D biosynthetic hydrogel system. *Biomaterials*. 2008. 29: 2597-2907

Poh M, Boyer M, Solan A, Dahl SLM, Pedrotty D, Banik SSR, McKee JA, Kinger RY, Counter CM, Niklason LE. Blood vessels engineered from human cells. *Lancet*. 2005. 265: 2122-2124

Rago AP, Dean DM, Morgan JR. Controlling cell position in complex heterotypic 3D microtissues by tissue fusion. *Biotechnology and Bioengineering*. 2009. 102(4): 1231-1241

Reyes CD, Garcia AJ. A centrifugation cell adhesion assay for high-throughput screening of biomaterial surfaces. *J Biomed Mater Res A*. 2003. 67(1): 328-333

Roger VL, et. al. Heart Disease and Stroke Statistics - 2011 Update. *Circulation*. 2011. 123: e18-e209

Rodriguez A, Alemparte MR, Baldi J, Navia J, Delacasa A, Vogel D, Oliveri R, Pereira CF, Bernardi V, O'Neill W, Palacios IF. Coronary stenting versus coronary bypass surgery in patients with multiple vessel disease and significant proximal LAD stenosis: results from the ERACI II study. *Heart*. 2003. 89: 184-188

Sabik III JF, Blackstone EH, Gillinov AM, Banbury MK, Smedira NG, Lytle BW. Influence of patient characteristics and arterial grafts on freedom from coronary reoperation. *J Thorac Cardiovasc Surg*. 2006. 131: 90-98

Schutte SC, Chen Z, Brockbank KGM, Nerem RM. Tissue engineering of a collagen-based vascular media. *Organogenesis*. 2010. 6(4): 204-211

Shi Z, Abraham G, Tarbell JM. Shear stress modulation of smooth muscle cell marker genes in 2-D and 3-D depends on mechanotransduction by heparan sulfate proteoglycans and ERK1/2. *PLoS ONE*. 2010. 5(8): e12196

Shi Z, Tarbell JM. Fluid flow mechanotransduction in vascular smooth muscle cells and fibroblasts. *Annals of Biomedical Engineering*. 2011. 39(6): 1608-1619

Shimzu N, Yamamoto K, Obi S, Kumagaya S, Masumura T, Shimano Y, Naruse K, Yamashita JK, Igarashi T, Ando J. Cyclic strain induces mouse embryonic stem cell differentiation into vascular smooth muscle cells by activating PDGF receptor B. *J Appl Physiol*. 2008. 104: 766-772

Solan A, Dahl SLM, Niklason LE. Effects of mechanical stretch on collagen and cross-linking in engineered blood vessels. *Cell Transplant*. 2009. 18(8): 915-921

Soletti L, Nieponice A, Guan J, Stankus JJ, Wagner WR, Vorp DA. A seeding device for tissue engineered tubular structures. *Biomaterials.* 2006. 27(28): 4863-4870

Soletti L, Hong Y, Guan J, Stankus JJ, El-Kurdi MS, Wagner WR, Vorp DA. A bilayered elastomeric scaffold for tissue engineering of small diameter vascular grafts. *Acta Biomaterialia*. 2010. 6: 110-122

Stegemann JP, Hong H, Nerem RM. Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol*. 2005. 98: 2321-2327

Sterpetti AV, Cucina A, D'Angelo LS, Cardillo B, Cavallaro A. Response of arterial smooth muscle cells to laminar flow. *J Cardiovasc Surg*. 1992. 33(5): 619-624

Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. *Am J Physiol Heart Circ Physiol*. 2005. 288: 1451-1460

Syedain ZH, Meier LA, Bjork JW, Lee A, Tranquillo RT. Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring. *Biomaterials*. 2011. 32(3): 714-722

Takeichi M. Functional correlation between cell adhesive properties and some cell surface proteins. *Journal of Cell Biology*. 1977. 75: 464-474

Tu JV, Pashos CL, Naylor D, Chen E, Normand S, Newhouse JP, McNeil BJ. Use of cardiac procedures and outcomes in elderly patients with myocardial infarction in the United States and Canada. *N Engl J Med*. 1997. 336: 1500-1505

Ueba H, Kawakami M, Yaginuma T. Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor-beta 1 and tissue-type plasminogen activator. *Arterioscler Thromb Vasc Biol*. 1997. 17(8): 1512-1516

Varani J, Bendelow MJ, Chun JH, Hillegas WA. Cell growth on microcarriers: comparison of proliferation on and recovery from various substrates. *J Biol Stand*. 1986. 14(4): 331-336

Warren L, Blithe DL, Cossu G. Variation of the carbohydrates of glycoproteins of cells growing on different surfaces. *J Cell Physiol*. 1982. 113(1): 17-22

Weber GF, Bjerke MA, DeSimone DW. Integrins and cadherins join forces to form adhesive networks. *Journal of Cell Science*. 2011. 124: 1183-1193

Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. *Science*. 1986. 231(4736): 397-400

Xue L, Greisler HP. Biomaterials in the development and future of vascular grafts. *J Vasc Surg*. 2003. 37: 472-480

Yamamoto K, Sokabe T, Watabe T, Miyazono K, Yamashita JK, Obi S, Ohura N, Matsushita A, Kamiya A, Ando J. Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. *Am J Physiol Circ Physiol*. 2005. 288: H1915-H1924

Youssef J, Nurse AK, Freund LB, Morgan JR. Quantification of the forces driving self-assembly of three-dimensional microtissues. *PNAS*. 2011. 108(17): 6993-6998

Zhang L, Su P, Xu C, Yang J, Yu W, Huang D. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett*. 2010. 32: 1339-1346

Appendix – Centritubing Results Presented by Experiment

A.1 – Spinning and Culture Observations During Centritubing

4.1 – Observing the Potential Causes of Tube Formation Inconsistency in the Centritubing System

Table 5 - Centritubing Results Batch 1.003

Tube #	Spinning Observations	Culture Observations	Tube Formation (2 days)
1.003-1	Smooth, uniform spin	Culture apparatus leaked	Dissociated tissue clumps
1.003-2	Motor came loose after 2 minutes, readjusted and started over	Culture apparatus leaked	Dissociated tissue clumps
1.003-3	Smooth, uniform spin	Culture apparatus leaked	Dissociated tissue clumps
1.003-4	Smooth, uniform spin	Culture apparatus leaked	Dissociated tissue clumps
1.003-5	Cap came off, added more cells, much motor vibration, large air bubble post-spin	Culture apparatus leaked	Dissociated tissue clumps
1.003-6	Cap came off, added more cells, motor came loose after 10 minutes, spun 10 more minutes after readjusted	Culture apparatus leaked	Dissociated tissue clumps

4.2 –Preventing Culture Apparatus Leaking and Motor Vibration

Table 6 - Centritubing Results Batch 1.005

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.005-1	Motor came loose after 10 minutes, added cushioning in clamps to hold motor securely	Sat 60 minutes before new media	Large dissociated tissue clumps
1.005-2	Smooth, uniform spin	Sat 45 minutes before new media, jostling and bumping to get spinning chamber into culture apparatus	Dissociated tissue clumps
1.005-3	Smooth, uniform spin	Sat 30 minutes before new media, slight jostling to get spinning chamber into culture apparatus	Dissociated tissue clumps
1.005-4	Smooth, uniform spin	Sat 15 minutes before new media, jostling slightly while removing spinning chamber cap	Large dissociated tissue clumps
1.005-5	Smooth, uniform spin	Much jostling and bumping to get spinning chamber into culture apparatus	Large dissociated tissue clumps

4.4 – Constraining Tissue Contraction, Improving Culture Ease of Use, and Observing Tissue Formation Over Time

Table 7 - Centritubing Results Batch 1.021

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.021-1	Smooth, uniform spin	Contraction began around 24 hours in culture	Tubular tissue construct formed around mandrel
1.021-2	Smooth, uniform spin	Contraction began around 24 hours in culture	Tubular tissue construct formed around mandrel
1.021-3	Smooth, uniform spin	Contraction began around 24 hours in culture	Tubular tissue construct formed around mandrel

Table 8 – Failed Cell Seeding for batch 1.023.

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.023-A	Cell suspension filled 95% of spinning chamber, bubbles during spinning	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber
1.023-B	Not centered perfectly, some wobbling during spinning	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber
1.023-C	Cell suspension filled 95% of spinning chamber, bubbles during spinning; not centered perfectly, some wobbling during spinning, but reattached for smooth, uniform spin	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber
1.023-D	Cell suspension filled 95% of spinning chamber, bubbles during spinning; not centered perfectly, slight wobbling during spin	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber
1.023-E	Smooth, uniform spin	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber
1.023-F	Cell suspension filled 90% of spinning chamber, added some media to fill; not centered perfectly, slight wobbling during spin	Silicone cap stuck to central mandrel, caused bumping and hitting cell-seeded wall when removed	Cells settled to bottom of spinning chamber

4.4 – Improving the Precision for Centritubing Manufacture and Preparation

Table 9 – Centritubing Results Batch 1.024.

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.024-A	Slight wobble, loss of ~10% volume during spin	Sat with 0.5 ml media for 15 minutes	Non-uniform contraction into ring and clumps
1.024-B	Good spin, loss of ~10% volume during spin	No complications	Non-uniform contraction into clumps

4.5 – Manufacturing for Cohesive and Uniform Tissue Formation

Table 10 – Centritubing Results Batch 1.025.

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.025-A Smoother Surface	Too much wobbling as soon as spinning began, so transferred cell suspension to new spinning chamber which had smooth, uniform spin	No complications	Tissue tube formed
1.025-B Rough Surface	Smooth, uniform spin	No complications	Some non-uniform contraction into clumps
1.025-C Rough Surface	Smooth, uniform spin	During cap removal, central mandrel came out but did not touch sides, it was inserted back	Non-uniform contraction into clumps

4.6 – Observing Repeatability in Tube Formation With a Smoother Seeding Surface

Table 11 - Centritubing Results Batch 1.026

Tube #	Spinning Observations	Culture Observations	Tube Formation (3 days)
1.026-A	Too much wobbling as soon as spinning began--reattached tighter and had smooth, uniform spin	No complications	Half of tissue tube formed (Figure 27B)
1.026-B	Smooth, uniform spin	No complications	Tissue tube formed
1.026-C	Smooth uniform spin, loss of ~10% volume during spin	No complications	Tissue tube formed (Figure 26A)
1.026-D	Alligator clips detached after a few seconds, but reattached and had smooth, uniform spin	No complications	Half of tissue tube formed (Figure 27A)
1.026-E	Smooth, uniform spin, loss of ~10% volume during spin	No complications	Tissue tube formed (Figure 26B)

4.6 – Observing the Repeatability of the Modifications to Centritubing

Table 12 - Centritubing Results Batch 1.028.

Tube #	Spinning Observations	Culture Observations	Tube Formation
1.028-A	Smooth, uniform spin	Central mandrel touching spinning chamber wall after spin, re-centered before culture	3 days - Tissue tube formed – poor harvest (Figure 29A)
1.028-B	Smooth, uniform spin	Central mandrel slightly attached to cap, cut loose with scalpel	3 days - Tissue tube formed – poor harvest (Figure 29B)
1.028-C	Smooth, uniform spin	Extended culture to 10 days	Tissue tube formed (Figure 30A)
1.028-D	Smooth, uniform spin	Extended culture to 10 days	Tissue tube formed (Figure 30B)

Table 13 - Centritubing Results Batch 1.030.

Tube #	Spinning Observations	Culture Observations	Tube Formation
1.030-A	Created bubbles during cell injection, added additional media; motor vibration after 8 minutes of spinning, changed batteries for smooth, uniform spin	No complications	Tissue tube formed (A)
1.030-B	Smooth, uniform spin	Central mandrel slightly attached to cap and hit cell-seeded wall, cut loose with scalpel	Tissue contraction in clumps (B)
1.030-C	Slight vibration of motor during spin, but spinning chamber had smooth, uniform spin	Central mandrel slightly attached to cap and hit cell-seeded wall, cut loose with scalpel	Tissue contraction in clumps (C)

Table 14 – Failed Cell Seeding for batch 1.031.

Tube #	Spinning Observations	Culture Observations	Tube Formation
1.031-A	Spinning chamber not filled completely, air bubble during spin	No complications	Cells settled to bottom of spinning chamber
1.031-B	1st spinning chamber had poor spin, so transferred to new chamber, created some bubbles during transfer	Central mandrel slightly attached to cap and hit cell-seeded wall, cut loose with scalpel	Cells settled to bottom of spinning chamber
1.031-C	Smooth, uniform spin	Central mandrel slightly attached to cap and hit cell-seeded wall several times, cut loose with scalpel	Cells settled to bottom of spinning chamber

A.2 – Tissue Sheet System

A.2.1 – Tissue Sheet Protocol

Tissue sheets and a method for their production were developed as a 2-dimensional model for centritubing. To seed and culture tissue sheets using the polycarbonate sheet molds, molds were first assembled to the desired configuration and autoclaved. Cells were resuspended to the desired concentration. Using sterile forceps, polycarbonate sheet molds were transferred to sterile 6-well culture plates. Two milliliters of cell suspension was pipetted into the seeding window of each mold and incubated at 37C and 5% CO_2 for 2 hours. Media was then added to each well until the mold was completely submerged. Molds were then returned to the incubator for culture.

Media was exchanged every 3-5 days. Media outside the molds was aspirated first, and then sterile forceps were used to tilt the polycarbonate sheet molds for easier aspiration of media from the mold without harming the tissue. Media was added to the mold seeding chamber gently and then the remainder of the well was filled to submerge the mold. The molds were then returned to the incubator.

To harvest sheets, the molds were disassembled and submerged in PBS. A combination of manipulating the tissue sheets with sterile forceps and fluid flow from a pipette were used to gently detach tissue sheets from the polycarbonate surface.

A.2.2 – Polycarbonate Sheet Mold Manufacturing

To create these tissue sheet molds (Figure 48) we used polycarbonate as the seeding surface to replicate a similar cell-surface interaction as in centritubing. The result was a simple two-piece system comprised of a solid seeding surface and a top that would constrain the cell suspension over the desired surface area.

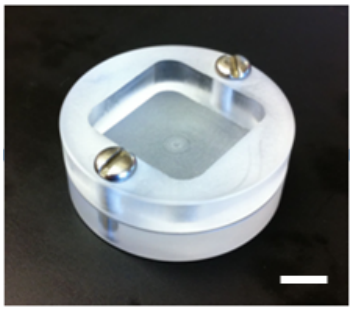

Figure 48 - Polycarbonate tissue sheet mold.
Scale bar ¼”

A 1.25-inch diameter polycarbonate rod was cut to 0.25-inch thick slices. Half of these were used as the bottom half of the mold. The other halves were used for the tops of the molds. Square holes (1.9cm x 1.9cm) were drilled through these slices using an end mill. Two smaller holes were drilled near the edges of each piece for screws to hold the two halves together. The molds were designed to fit within a well of a 6-well plate.

Assembly and Anchors

After several tests, it was determined that anchors were needed to prevent contraction and retain the tissue sheets in the desired shape and size after culture. Several mesh materials were cut to ring-like squares with outer dimensions of 2 cm by 2 cm and a thickness of 4 mm. The materials used to make anchors are listed in Table 15. Anchors were centered on the seeding surface before mold assembly so they would be constrained between the two pieces of the mold. To prevent leaking of cell suspensions, vacuum grease was coated around the outer edge of the molds. The components of the tissue sheet system are shown in Figure 49.

Table 15 - Table of anchors materials and pore sizes.

Name	Material	Pore Size (um)
Vyon	PE	38
Nitex	Nylon	300
PE Sheets	PE	90-130

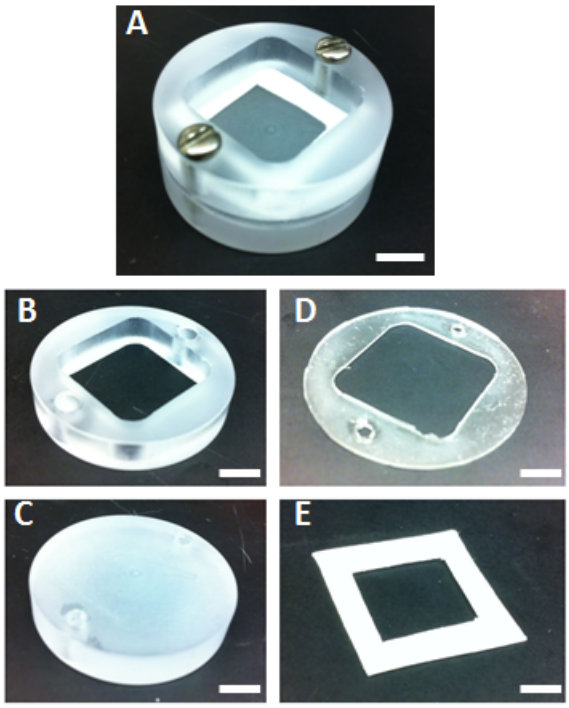

Figure 49 - The components of the tissue sheet system. The sheet system (A) included the seeding well (B), the seeding surface (C), the PDMS seal (D), and the anchor (E). Scale bars ¼"

A.2.3 – Tissue Sheet Culture as a 2-D Model for Centritubing

This section describes the first step in modeling centritubing using tissue sheets: determining the best method for creating sheets, while retaining similar environmental cues to the spinning chamber. First, we needed to learn the typical time requirement for tissue sheet formation using raSMCs.

Creating Tissue Sheets: Proof of Concept

The objective of this study was to determine how long it takes for sheets to grow using a range of cell densities. Standard 24-well tissue culture plates were used as a seeding surface for tissue sheets. raSMC concentrations of 25,000 cells, 50,000 cells, 100,000 cells, 200,000 cells, 500,000 cells, and 1,000,000 cells were prepared and seeded on two separate 24-well plates. These cell amounts equate to cell densities of 125 cells/mm^2, 250 cells/mm^2, 500 cells/mm^2, 1000 cells/mm^2 (100% confluence), 2500 cells/mm^2, and 5000 cells/mm^2 (approximately 5 cell layers). One plate was seeded with three wells of each concentration and then centrifuged for 15 minutes at 1550 rpm, then set in the incubator to culture. The second plate was seeded with two wells of each concentration and then set at room temperature for 15 minutes before being set in the incubator for culture.

The plates were observed every 24 hours and examined for tissue sheet formation. After eight days, they had visibly formed sheets (Figure 50). Every well had a sheet formed in it and some had begun to contract around the edges. It appeared that the wells that were seeded with more cells were a

bit thicker and more contracted, but no quantification of this observation was performed. It is also possible that tissue formed before previously noted, but could not be seen because it was uniform throughout the well. Sheets were left in culture up to ten days.

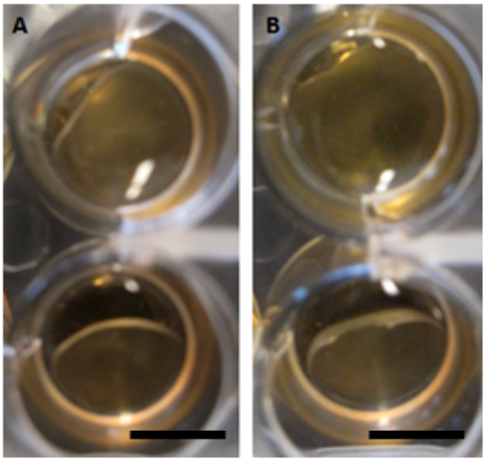

Figure 50 - Tissue sheets on culture plastic.
Sheets were cultured for 10 days after static seeding (A) and centrifugal force seeding (B) exhibited no significant differences in terms of tissue formation time and contraction. Scale bars 1 cm

These tests suggested that an adhesive surface is needed to form sheets and that centrifuging will still allow for sheet formation and can be used to model centritubing. However, much about using a standard tissue culture plate is different from centritubing—material, cell adhesion properties, and surface shape. Therefore, a more representative method was developed for tissue sheet formation using a polycarbonate surface and a surface area similar to that in the spinning chamber in centritubing.

Polycarbonate Molds for Tissue Sheets: Proof of Concept

The objective of this study was to provide a Proof of Concept for using polycarbonate molds to grow tissue sheets. Molds for tissue sheets (Figure 51A) were manufactured as described in Appendix section A.2.2. These molds were designed to have a seeding surface area similar to that of the spinning chambers used in centritubing. The polycarbonate sheet molds had a surface area of 3.61 cm^2 and the polycarbonate spinning chambers had a surface area of 3.79 cm^2. This provided an easier comparison between the two systems.

Three polycarbonate sheet molds were prepared and sterilized, then seeded with $1x10^6$ raSMCs (2770 cells/mm^2) each and cultured in a 6-well plate in the incubator.

After four days one mold was tested for tissue formation time. The top of the mold was removed and a layer of tissue was observed in the shape of the opening (Figure 51C). Trypan blue was used to determine cell viability within the tissue as follows. Trypan blue was pipetted over the tissue

sheet. After three minutes the trypan blue was washed off with PBS and the tissue was not stained blue. Next, 70% ethanol was pipette onto the tissue and removed after three minutes. The tissue was once again stained with trypan blue for three minutes and washed with PBS. This time the tissue stained a dense blue. The polycarbonate mold with the attached tissue was submerged in fresh PBS and forceps were used to gently scrape the tissue sheet away from the mold. The sheet remained a cohesive tissue during harvest (Figure 51B).

The remaining tissue sheets were cultured until day 7. These sheets were also stained with trypan blue similar to the day 4 sheet, but a few small holes in the tissue were observed. These were removed from the molds using a different method. The molds were submerged in PBS and a combination of gentle scraping with forceps and fluid flow from a 1000 µl micropipette were able to detach the tissue in cohesive sheets.

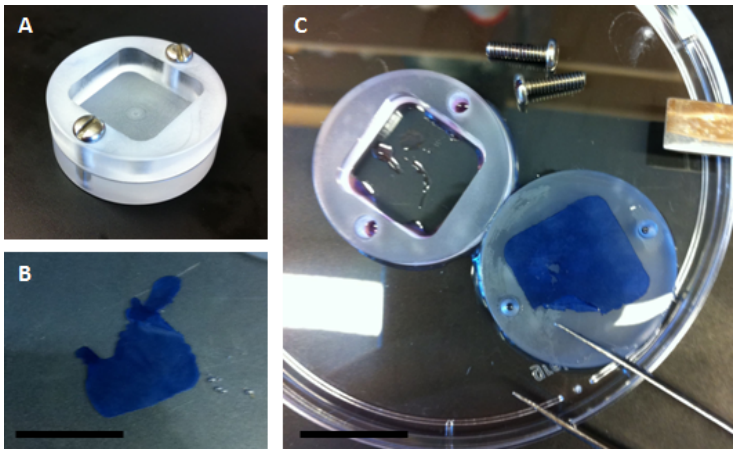

Figure 51 - Customized polycarbonate sheet molds. (A) Sheet molds were used to create 1.9x1.9 cm tissue sheets (B), which could be harvested from the molds using forceps and PBS (C). Scale bars 1.9 cm

Overall, it was determined that raSMC tissue sheets could be grown on tissue culture plates or the customized polycarbonate molds, but not on agarose surfaces. The polycarbonate sheet molds more closely replicated the environment in centritubing and more were manufactured to move forward with larger studies.

A.2.4 – Mechanical Testing of Tissue Sheets

The objective of this study was to determine if tissue sheets could be used for burst pressure testing, which is the primary mechanical consideration for tubular tissues. If these tissue sheets could be used for burst pressure testing, it would allow a calculation of tissue strength that could be translated back to centritubing. For example, such a test could be used to determine the ideal media formulation to improve tissue strength in centritubing without the actual risk of confounding data in the complex and inconsistent centritubing system.

A burst pressure test of tissue sheets can be performed by a membrane inflation device (Billiar, 2005). The membrane inflation device would seal the tissue sheet over a saline outlet. The device would fill the sealed bubble of tissue with saline until it burst while measuring the pressure within the system and the expansion of the tissue. This test is the tissue sheet equivalent to burst testing, which is the primary measure of strength in tubular tissue constructs.

To test this, one tissue sheet was not fixed immediately, but was kept in PBS at room temperature. The tissue sheet was floated in PBS onto the membrane inflation device, however due to contraction during and after removal the tissue sheet had shrunk slightly from the size at which it grew on the mold. The tissue sheet was designed to be large enough to easily perform membrane inflation, however at this shrunk size it would be difficult to get it attached exactly onto the device. Furthermore, the tissue sheet was floppy and would fold upon itself easily, making it difficult to handle and arrange correctly on the membrane inflation device. These tissue sheets could still be used for membrane inflation if they were constrained to the intended shape and size.

A.2.5 – Identifying Anchor Materials for Preventing Contraction in Tissue Sheet Culture

After observing difficulties in preparing tissue sheet samples for membrane inflation testing, it was determined that an anchor system should be developed to constrain the tissue sheets in size and shape. Anchors have been used previously for constraining tissue sheets (Gauvin, 2011). Several different materials with varying pore sizes were investigated for use as anchors. Six polycarbonate sheet molds were prepared and sterilized. Two molds utilized vyon as anchors, two molds utilized nitex as anchors, and two molds utilized PE sheets as anchors. raSMCs were seeded into molds at a concentration of $2x10^6$ cells/mold in a 6-well plate. After 3 hours of static seeding, wells were filled with growth media to submerge molds.

After four days, culture was terminated. Tissue sheets with PE sheets or vyon as anchors had grown into small clumps of tissue (Figure 52 A, B). Tissue sheets with nitex as anchors looked promising, but were not uniform and showed uneven contraction (Figure 52 C, D).

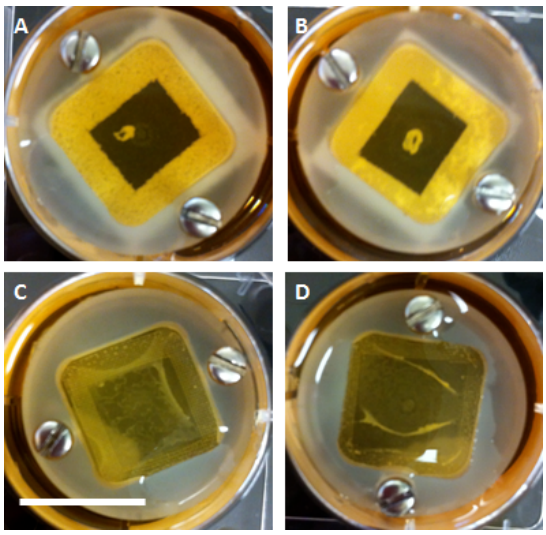

Figure 52 - Tissue sheets grown on anchors. Tissue on PE sheet anchors (A) and vyon anchors (B) contracted into balls within 4 days of culture in growth media. Tissue sheets grown on nitex anchors (C, D) did grow into the anchors, but exhibited non-uniform contraction and ripping. Scale bar 1.9 cm.

A.2.6 – Identifying Methods for Seeding Tissue Sheet Molds by Centrifugal Force

In order to truly model centritubing in the tissue sheet system, centrifugal force must be used to seed raSMCs onto the polycarbonate molds. This experiment examined various methods for sealing the polycarbonate molds so cells and media would not leak out between the top and bottom pieces during centrifuging.

Six different arrangements were used to test if cells could be seeded onto the polycarbonate sheet molds using centrifugal force. First, $1x10^6$ raSMCs in 2 ml growth media were centrifuged in a polycarbonate sheet mold. Second, 2 ml growth media was centrifuged in a polycarbonate sheet mold. Third, 2 ml growth media was centrifuged in a polycarbonate sheet mold with a square of PDMS clamped between the top and bottom pieces. Fourth, 2 ml growth media was centrifuged in a polycarbonate sheet mold with a square ring of filter paper clamped between the top and bottom pieces. Fifth, 2 ml growth media was centrifuged in a polycarbonate sheet mold with a square ring of PDMS and a square ring of filter paper clamped between the top and bottom pieces. Sixth, 2 ml growth media was centrifuged in a polycarbonate sheet mold with silicone vacuum grease coated around the edges where the top and bottom pieces meet. All arrangements were placed in a well of a 6-well plate and centrifuged at 1500 rpm for 5 minutes (n = 1 each).

The plausibility of using each arrangement for seeding cells by centrifuging was determined by observing media leakage out of the mold. As seen in Table 16, only the PDMS ring with the filter paper

ring showed no leakage of media during centrifuging. We assume that a PDMS ring without the filter paper would also be adequate; however, anchors may be necessary for easier handling of tissue sheets after culture. Vacuum grease may be used to coat the outside for static seeding experiments only to ensure no leakage occurs.

Table 16 – Centrifugation of water in sheet molds.

Arrangement	After Spin	Plausible?
Just mold with cells	All leaked, cells in well	No
Just mold	All leaked	No
PDMS square	Little leaked	No
Filter paper ring	All leaked	No
PDMS & filter paper rings	**None leaked**	**Yes**
Vacuum grease	Most leaked	For static seed